University of Alberta

Mutant Group B Streptococcus surface expressed Phosphoglycerate kinase (PGK) with reduced plasminogen binding

by

Khalida Siddiqua

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Sciences - Laboratory Medicine and Pathology

Spring 2014
Edmonton, Alberta

Abstract

Group B streptococcus (GBS) is a Gram-positive streptococcus bacterium that can cause severe invasive disease in the human neonate. This can manifest as pneumonia, septicemia and meningitis. While antibiotic prophylaxis has reduced the incidence GBS disease in the neonatal population, it is still the one of the leading cause of neonatal invasive disease in North America including Alberta. Phosphoglycerate kinase (PGK), a glycolytic enzyme, has been demonstrated to be on the surface of GBS. Surface expressed GBS-PGK interacts with the host-cell protein plasminogen, which is thought to help bacteria invade further into the host by destruction of host barriers. In this thesis, a triple mutant GBS-PGK molecule PGK-M9 was created based on information from a space-filled model of PGK-plasminogen interaction sites. PGK-M9 bound 95% less plasminogen than the wild type GBS-PGK. This mutant will permit further investigation into the role of surface GBS-PGK *in vivo* models of GBS infection.

Acknowledgements

Graduate school has been a life changing experience for me. I learnt a lot about failures and success and the true spirit of hard work. I would like to thank the Almighty for giving me this opportunity. In this journey there are many people that I would like to thank. Firstly, I would like to thank my supervisor Dr. Greg Tyrrell, for this continued support and encouragement. I cannot thank him enough for always believing in me, and letting me learn from my mistakes. This allowed me to grow as an individual and realise my strengths and weaknesses. I think my graduate thesis would not have been possible without the training and contributions provided by Dr. Tyler Boone. I cannot thank him enough for allowing me to build on this work. I would also like to thank all the post-docs, and fellow researchers from whom I have learnt a lot.

Secondly, I would like to thank my supervisory committee Dr. Monika Keelan, and Dr Linda Chui. Special thanks for all the time and the helpful suggestions they have given. I would also like to thank the entire LMP department for their support, especially Cheryl Titus. The most enjoyable experience of my graduate career was the graduate teaching and learning program. I would like to thank Kristi Lew my teaching mentor for all her guidance in helping me develop my teaching skills and for providing the teaching opportunities.

Last, but never the least, I would like to thank my parents, husband and brother. My parents have always been a strong pillar of support. I could not have gotten this far without all the sacrifices they made. They immigrated to Canada to ensure that I received the best education. I know they will feel accomplished upon the completion of my graduate school. Special thanks to my husband for all this love and support especially during the thesis writing process. He was always there to help me out. I would also like to thank my in-laws for their continued support.

Table of Contents

Table of Contents

List of Tables

List of Figures

Chapter 1:

Introduction

Group B *streptococcus* (GBS) is a Gram-positive streptococcus bacterium that can cause severe invasive disease in the human neonate. This can manifest as pneumonia, septicemia and meningitis. The primary route of pathogenesis in neonates requires GBS to be inhaled from infected amniotic fluid into the lungs followed by invasion of the lung epithelium further into the blood where the bacteria can gain access to the meninges. GBS can then cross the blood-brain barrier, causing meningitis (1). While antibiotic prophylaxis has reduced the incidence of GBS disease in the neonatal population, it is still the one of the leading causes of neonatal invasive disease in North America, including Alberta (2), Table 1.1. The molecular mechanisms involved in invasive GBS disease are not completely understood. Phosphoglycerate kinase (PGK), a glycolytic enzyme, has been shown on the surface of GBS (3). Further, Boone et al. (4, 5) demonstrated that surface-expressed GBS-PGK interacts with the host-cell protein plasminogen. Plasminogen binding is seen in many bacteria (4-8) and fungi (9). Plasminogen binding destroys host barriers, which is thought to help these organisms invade further into the host. Through peptide mapping and site-directed mutagenesis, Boone et al. (4) have identified critical amino acids in the GBS-PGK molecule that are necessary for binding PGK to plasminogen and actin. Boone et al. used site-directed mutagenesis to create a mutant GBS-PGK molecule that displayed reduced plasminogen-binding; this mutant was designated mutant PGK-M8 (4, 10). My thesis project involved using the PGK-M8 molecule as a starting point to create a GBS-PGK molecule that does not bind to plasminogen. Although PGK-M8 has been shown to have reduced binding to plasminogen, a

mutant GBS-PGK molecule that completely abrogates plasminogen binding has not been created. The objective of my project was to create a GBS-PGK molecule that was glycolytically active and yet had lost the ability to bind human plasminogen. The creation of this tool can be used to enhance our understanding of the role of GBS-PGK with respect to plasminogen binding in GBS pathogenesis.

1.1 **Group B streptococcus**

The Group B *streptococcus* (GBS) or *Streptococcus agalactiae* belongs to the family Streptococci. It is a facultative anaerobic Gram positive coccus that grows in chains (11). Streptococci are classified using the Lancefield classification scheme. This classification was developed in the 1930's by Rebecca Lancefield (12). Using this classification scheme, streptococci can be grouped as A, B, C, D, F, G and N. The different groups are based on the different carbohydrates found in the streptococcal cell wall. The streptococci that are considered human pathogens belong to groups A, B and D. *Streptococcus pyogenes belonging to group A are also known as* Group A *streptococcus* (GAS). GAS and GBS are examples of human pathogenic streptococci. GBS possess a polysaccharide capsule. There are 10 antigenically different GBS capsule types and each one is referred to as a serotype. The different GBS serotypes are Ia, Ib, II, III, IV, V, VI, VII, VIII, IX. The GBS serotypes that cause the majority of human disease are Ia, Ib, II, III and V (11, 13-15).

1.1.1 *Group B Streptococcus Disease Manifestation*

1.1.1.1 GBS disease in neonates:

Since the 1970's, GBS has been known to cause invasive disease in neonates. For the neonate, invasive GBS disease can be divided into two presentations: early onset disease (EOD) and late onset disease (LOD). EOD is classified as occurring at 24 hours to 7 days of age. EOD is typically expressed as pneumonia which may progress to sepsis. LOD is classified as occurring beyond 7 days and up to 3 months of age**.** LOD can be expressed as a bacteremia, but in > 50% cases it presents as neonatal meningitis. The main route of GBS infection in neonates is the uptake of maternal GBS from colonized mothers. Studies have shown that approximately 10-30% women in North America have asymptomatic GBS rectal and vaginal colonization (2). During pregnancy these colonized women may pass the GBS to their babies either *in utero*, or while the neonates are passing through the birth canal (1). GBS may ascend up from the maternal vagina and invade the placental membranes and amniotic fluid. The neonates may then aspirate the GBS from the contaminated amniotic fluid into their lungs. The first sign of GBS infection may present as pneumonia. The bacteria can further invade tissues in the lungs, causing epithelial and endothelial cell destruction, leading to sepsis. In some cases, GBS may also invade the blood-brain barrier, causing meningitis, which may lead to death (1, 13). In the United States (US), in the early 1990s, the rate of GBS disease was 1.7 cases per 1000 live births (2). In 1996, the Center for Disease Control and Prevention (CDC) guidelines for screening for maternal colonization and maternal antibiotic prophylaxis were

implemented and this led to a decrease in GBS disease in neonates (Figure 1.1). The details about the screening and prevention guidelines for EOD will be discussed later. The rate of GBS disease decreased further to 0.34-0.37 cases per 1000 live births in the late 2000s after the introduction of universal screening for maternal GBS colonization and intrapartum antibiotic prophylaxis (Figure 1.1) (2). However, the maternal antibiotic prophylaxis and maternal screening for GBS colonization have not impacted the rate of LOD in the US. LOD remained the same at 0.4 cases per 1000 live births from the 1990s to 2008 (2) (Figure 1). A recent study of LOD cases in 322 NICUs in the US showed that the GBS LOD rate had increased from 0.8 per 1000 cases in 1997-2001 to 1.1 per 1000 cases in 2002-2010 (16). The authors indicate that this increase in LOD occurred after the introduction of maternal intrapartum antibiotic prophylaxis (IAP) in 2002. The Alberta rate is comparable to the CDC GBS EOD rate, suggesting little variation in disease rates between Canada and the US. The GBS EOD rates in Alberta were 0.34 per 1000 live births in 2010 and 0.28 per 1000 live births in 2011. The GBS LOD rates in Alberta also increased, from 0.26 per 1000 live births in 2010 to 0.30 per 1000 live births in 2011 (Table 1.1). This data on GBS EOD and LOD rates in Alberta was collected electronically from the bacterial typing unit surveillance where isolates causing invasive GBS disease were submitted for serotyping. Understanding the GBS disease pathogenesis and virulence factors is essential in further reducing both GBS EOD and GBS LOD.

From a disease progression perspective, in summary (Figure 1.3), GBS disease in neonates begins with 1) the GBS ascending from the vaginal area of a

colonized pregnant mother and 2) crossing the placental membranes and causing *in-utero* infection. 3) The neonate breathes in the GBS during the birth process or *in utero,* causing pneumonia. 4) The GBS is able to invade further into the bloodstream, causing septicaemia. 5) GBS can also invade the neonatal blood-brain barrier, causing meningitis (1).

1.1.1.2 Invasive GBS Disease in adults

GBS disease in adults is mainly described in pregnant women and non-pregnant adults. In pregnant women, GBS can cause infection prenatally, during pregnancy and postpartum. Clinically it can present as endometritis, an intra-amniotic infection, vaginitis, urinary tract infections and, sometimes, as sepsis (17), (18). The adults most at risk of invasive GBS disease are the elderly, over the age of 65 (19). The incidence of GBS disease in non-pregnant adults has increased greatly since 1990. According to a study done in 10 states in the US, GBS disease in non-pregnant adults doubled from 3.6 cases per 100,000 in 1990 to 7.3 cases per 100,000 in 2007 (19). The serotypes most commonly associated with invasive GBS disease in adults are serotypes Ia, III and V (13, 14). Studies done in the US have shown that the majority of invasive GBS infections occur in elderly adults over the age of 65, and that more than 50% of GBS disease-caused mortality occurs in the elderly (15). Having comorbid diseases like diabetes mellitus, cancer, stroke or cirrhosis of the liver (15, 20), and being bedridden in nursing homes are also important risk factors (15). Another factor that has been shown to cause GBS disease in elderly adults already colonized with the

bacterium is altering the integrity of mechanical barriers by surgery or cystoscopy, or by inserting a urinary catheter (13,15). Lastly, the diminished immune response in elderly adults has been found to promote GBS disease (15). Clinical manifestations of the disease in adults include soft tissue and skin infections, bacteremia and osteomyelitis and, rarely, endocarditis (20-22). In an institution in Germany, GBS was found to be the main cause of "perianal" dermatitis in elderly adults (23) .

1.2 GBS Disease Pathogenesis

There are a variety of structures in GBS that allow it to invade the human host. The principle sites that GBS targets are the following: colonization of the vaginal area by epithelial attachment; invasion of the respiratory epithelium in neonates, causing pneumonia; resistance to the host immune system, causing septicaemia; and invasion of the endothelial blood-brain barrier, causing meningitis. Each of these events will be discussed in detail in the forthcoming sections.

1.2.1 Attachment to host cells

Attachment to host cells is the initial stage of GBS infection and host cell invasion. GBS were recently found to express pili on their surface (24). The pili contain three proteins: PilA and PilC help in host cell adherence, and PilB forms the pili backbone (25, 24, and 26). PilA was found to have a von Willebrand adhesion domain which was shown to play a role in pilus associated adhesion by GBS (27). Studies have shown that mutating the GBS pili proteins inhibits the

attachment of respiratory epithelial cells *in vitro*; the mutant strain lacking the von Willebrand adhesion domain did not adhere to the respiratory epithelial cells (27, 28). One of the key steps in host-cell invasion is when GBS binds to the extracellular matrix proteins. The GBS cell surface proteins bind to the eukaryotic proteins fibrinogen, fibronectin and laminin in the ECM (extra cellular matrix) of host cells (26), (29), and (30). Fibrinogen binding is mediated by the surface GBS protein FbsA and FbsB (30). In addition, ScpB, a GBS surface protein, also binds fibronectin through a fibronectin binding domain (C-terminal) (26), (31). Besides binding to fibronectin, ScpB also binds to integrins through an arginine-glycine-aspartate (RGD) region found in the N-terminal domain of the ScpB protein (32). It is interesting to note, however, that *in vitro* GBS does not bind to the fibronectin present in all types of cell lines. Tyrrell *et al* showed that GBS did not bind and invade MRC-5 cells (a type of cell line rich in fibronectin); however, it was able to bind and invade HeLa cells which had less fibronectin than MRC-5 cells (33). This suggests that various cell types may express a different form of fibronectin that the GBS fibronectin-binding proteins do not bind to. In addition to FbsA/B and ScpB, the alpha C protein (αCP) found on the GBS surface has been shown to help in the invasion of epithelial cells. The αCP protein binds to the glycosaminoglycan (GAG) region found on the epithelial cell surface (34). Studies have shown that GBS strains expressing a αCP variant invaded the human cervical epithelial cells (ME180) less efficiently (26). Another mechanism of GBS invasion involves wild-type αCP binding to integrins α1β1 on the host epithelial cell. The D1 domain of the N-terminal region of αCP

binds to the integrins, and causes integrin clustering in ME180 cells; a mutation in the D1 region inhibited the invasion of GBS into ME180 cells (35). The binding of host cell integrins by ScpB and αCP GBS proteins has been discussed above. The use of integrins gives rise to the host-cell signalling pathway that leads to actin-cytoskeletal re-arrangements (35). Previous researchers in our laboratory have found that phosphoglycerate kinase (PGK) present in the GBS cytoplasm is also present on the GBS cell surface (5). The GBS-PGK has been shown to bind plasminogen and actin, and can cause actin cytoskeleton rearrangements when expressed inside the host cell (5). Another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is also involved in binding the GBS to the host-cell plasminogen and activating the plasmin to degrade the cellular matrix proteins (36). Binding the GBS surface glycolytic enzyme PGK to the host plasminogen and plasmin activation will be discussed in later sections. In conclusion, GBS uses a variety of mechanisms to attach to host cells, including GBS pili proteins PiliA and PiliB; the GBS surface proteins αCP and ScpB; and the GBS surface glycolytic enzymes GAPDH and PGK.

1.2.2 Invasion of Host - Cellular Barriers

After initially adhering to the host cell, GBS is then able to invade and cause infection. This section will focus on the mechanisms that GBS uses to invade host cell. It involved the GBS ascending from the vagina of colonized, pregnant woman into the placenta of her infant. GBS can invade the placenta by secreting a protease which is encoded by the gene *hylB*. This enzyme acts as a

hyluronidase and cleaves the hyaluronic acid molecule found in the placenta and amniotic fluid (37, 38).

Actin polymerization has been shown to be an important event in the GBS invasion of the host cell. I*n vitro* GBS invasion of respiratory epithelium and actin polymerization were reduced greatly by the treatment of the respiratory epithelium cell line by an actin inhibitor cytochlasin (39). In addition, *in vitro* GBS invasion of the cervical epithelium (HeLa cell line) also needs actin polymerization (33) and the treatment this cell line by an actin inhibitor cytoclasin reduced GBS invasion greatly. As discussed previously, both the ScpB and αCP GBS proteins cause the host cell integrins to be activated and help the GBS to invade by activating the actin-cytoskeletal rearrangement pathway. Also, the αCP of GBS binds host α1β1 integrin, which promotes the *in vitro* internalization of GBS within human epithelial cells (35). The host cell actin-cytoskeletal rearrangement pathway activated by GBS may be integrin-dependent. Integrin-linked kinase (ILK), a serine threonine protein kinase, has been shown to interact with the host cell cytoplasmic tails of β integrin. The integrin-linking kinase (ILK) has been shown to be involved in the Group A *streptococcal* model of invasion into epithelial cells. It was determined that fibronectin-binding proteins (M1 or PrtF) found on GAS bind to fibronectin, which leads to α5β1 integrins clustering (40). The involvement of ILK in GBS invasion could be investigated in the future as it has already been shown that the αCP of GBS causes α1β1 integrin clustering (35).

In addition to ILK, focal adhesion kinases (FAK) have also been shown to be needed for GBS invasion. In eukaryotic host-cells, the focal adhesions (Fas) are centres containing many signalling proteins, and together they form a link between the extracellular matrix and the cytoplasm of the cell (41, 42). Fas consist of integrins, the adaptor proteins like talin and paxillin (these bind to the intraceulluar domain of integrin), and signalling proteins like FAK. FAK binds to the integrin-adaptor protein complex (also called the adhesion complex) and, the integrin-adaptor protein and FAK together form the focal adhesions (41-43). FAK is a 125-kDa protein tyrosine kinase (PTK). It was named focal adhesion kinase because it localizes to focal adhesions in the cell and transmits signals from the focal adhesions to other signalling molecules downstream (42, 44). The N-terminal domain of FAK contains a catalytic tyrosine site Tyr-397 that helps to auto–phosphorylate FAK (45), and contains a FERM domain (46).The FERM domain is a region of 300 amino acids. Its function is to facilitate protein-protein interactions in the cell (47). It also helps FAK to interact with integrin by binding to the β1 integrin subunit tail in the cytoplasm (48, 49). The FERM domain regions are essential for FAK signalling. A mutant with the mutated FERM sequence was shown to have reduced tyrosine phosphorylation of FAK, making the FAK less able to transmit protein signals downward (46).

Phosphatidylinositol 3-kinase (PI3K) (a signal transduction enzyme) was shown to bind to activated FAK. Inhibiting PI3K also inhibited cell migration caused by FAK (50). ILK has also been shown to interact with phosphoinositide-3 kinase (PI3K) (40, 51). Earlier in this chapter, it was discussed that when GBS

binds to the host cell, PI3K is activated (33, 52). PI3K is a lipid kinase molecule found in the eukaryotic cells, and it regulates the actin cytoskeleton. PI3K activation via GBS has been shown to be involved in actin cytoskeleton rearrangements (53). The phosphorylated PI3K molecule leads to the formation of the 3, 4- bisphosphate (PIP_2) molecule. The PIP2 molecule is then phosphorylated, and forms the 3, 4, 5-triphosphate (PIP_3) molecule. The PIP3 leads to the phosphorylation and activation of the protein kinase B (Akt) (54) (Figure 1.4). Akt affects other regulators in various pathways downstream, and these pathways play a role in cell survival, proliferation, differentiation and migration (53). Activating Akt may help GBS survive in the host cell, as Akt inhibits the host-cell pro-apoptotic signals (55). Glycogen synthase kinase-3 (GSK-3) is also phosphorylated downstream of Akt upon GBS infection (53). GSK-3 is a serine/theorine kinase that controls cell apoptosis (56, 57); phosphorylation of GSK-3 inhibits GSK-3 and thereby prevents host-cell apoptosis (58, 53). In addition to activating Akt, PIP3 also activates Rac 1, a molecule that belongs to the family of Rho GTPases. Rho family GTPases are signalling molecules found in the eukaryotic cytoplasm and have been shown to be involved in cytoskeletal rearrangement. Rac 1 has been shown to cause actin polymerization, which helps in the motility of the actin cytoskeleton (59, 60). As previously indicated, actin polymerization is important for GBS invasion of epithelial cells. Our laboratory has previously shown that the GBS infection of epithelial cells increases activated Rho family GTPases Rho A, Rac 1 and Cdc42, and that treating HeLa cells with pan-GTPase inhibitor inhibited GBS invasion

(61). We can deduce that GBS may cause PIP3 to form in the host cell, which activates the Rac1; the activated Rac1 can cause actin polymerization and motility of the actin cytoskeleton, which helps the GBS invade the host cell. Additionally, actin cytosketal rearrangements have been shown to be involved in GBS invasion of endothelial cells. GBS meningitis in neonates is caused when the blood-brain barrier made up of microvascular endothelial cells is breached. *In vitro* experiments involving the GBS invasion of HBMEC (human brain microvascular endothelial cells) showed that cell lysates, when blotted, show enhanced FAK phosphorylation, indicating that the actin cytoskeleton pathway has been activated (62). Phosphorylation of FAK upon GBS infection has been shown to induce its association with PI3K and paxillin thereby causing actin polymerization and the GBS invasion into host endothelial cells (62). In addition, GBS invasion of HBMEC was inhibited when PI3K and tyrosine kinases were inhibited. Therefore in conclusion, a simplified possible pathway of GBS invasion of host epithelial cells based on the previously discussed *in vitro* studies could be the following: 1) GBS αCP binds to the epithelial cell via the α1β1 integrin. 2) The FAK/ILK gets phosphorylated, causing the paxillin adaptor protein to attach, forming an adhesion complex which binds the integrin, activating the focal adhesions. 3) The FAK/ILK interacts with the PI3K and activates the PI3K/AKT pathway and /or PI3K activates Rac 1. Both these pathways can cause actin polymerization and actin cytoskeleton motility. 4) Lastly, the host actin cytoskeletal motility may cause the GBS internalization into the host cell (Figure 1.4).

1.2.3 Host Immune System Evasion

GBS that invades the host-cell barriers can survive by avoiding the host immune attack and disseminating through the host immune system. A variety of GBS virulence factors allow the GBS to evade the host-immune system. One such factor is the GBS capsule, which contains sialic acid. Sialic acid is also found in host eukaroytic cells, and therefore the GBS capsule is not recognized by the host's immune system (63). Because the immune system doesn't recognize the capsule, the host's alternative complement pathway isn't activated, which protects the GBS from phagocytosis (64, 65). A study done in the early 1990s showed that immediately after inoculation, more encapsulated GBS strains accumulated in the neonatal alveolar macrophage than non-encapsulated GBS strains. This suggests that GBS strains with capsules evade being phagocytized in the neonatal lungs, providing them with an advantage to spread further from the neonatal lungs (66). It has also been shown that the GBS strains that lack capsular polysaccharides do not lead to sepsis (66, 67, and 68). In a recent study, both the unencapsulated GBS mutant and encapsulated GBS were internalized into antigen-presenting dendritic cells *in vitro*, but the unencapsulated GBS strain was killed at a faster rate compared to the encapsulated GBS strain (63). Therefore, the GBS capsule is one of the factors essential for host-cell immune evasion, allowing the GBS to disseminate and cause infection. As discussed previously, ScpB is an adhesion protein that helps GBS internalize into the epithelial cells (69). ScpB is also called C5a peptidase, as it can also cause proteolytic breakdown and inactivation of human C5a, a human complement component that is needed for neutrophil recruitment (67). The breakdown of C5a

thereby reduces host neutrophil chemotaxis at the site of infection (26). Another host immune system component that GBS is able to evade is the host cell macrophage. After GBS invades, the host-cell macrophages produce reactive oxygen species to cause oxidative stress. GBS is able to produce superoxide dismutases enzymes (SodA), which prevent the toxic effects of the reactive oxygen species produced by the macrophages. GBS SodA is similar to the catalase enzyme of staphylococci and converts the reactive oxygen anions (O_2^-) into oxygen atom (O_2) and hydrogen peroxide (H_2O_2). O_2 and H_2O_2 are further metabolized into enzymes catalase and peroxidase, reducing oxidative stress in GBS (53, 67).

As discussed previously, GBS LOD can clinically present as sepsis and, sometimes, as meningitis. Septic shock in neonates can be fatal. This event may start with GBS infection, which produces host cytokines, leading to inflammation (70). A study (70) done using cord blood monocytes showed that GBS activates the mitogen-activated protein kinase (MAPK) signalling pathway that activates transcription factors. The enhanced expression of nuclear factor kappa light chain (NF-kB), an activator protein 1(AP-1) that enhances activated B cells, was shown to regulate the production of cytokines and has been linked to GBS septic shock (70).

In conclusion, GBS uses sialic acid-rich capsular polysaccharide, C5a peptidase and SodA to evade the host immune system. The MAPK signalling pathway has been shown to be activated in GBS LOD, causing enhanced expression of NF-kB and AP-1 which has been linked to GBS septic shock.

1.3 Prevention and Treatment Protocols

EOD caused by GBS can be controlled by screening pregnant mothers to see if they are carrying GBS. Vaginal and rectal culture swabs to screen for GBS colonization are indicated for all pregnant women at 35-37 weeks gestation; this is called universal screening of pregnant women for GBS colonization (2) and is recommended by Money *et al*. and The Infectious Diseases Committee of the Society of Obstetricians and Gynaecologists of Canada (SOGC) (71). If they test positive, they can be treated with intrapartum antibiotic prophylaxis. In Canadian hospitals, the guidelines for screening pregnant women for GBS colonization are similar to CDC recommendations. Both the CDC and Money *et al.* recommend intrapartum antibiotic prophylaxis in the following situations by: 1) the vaginal and rectal swab done between 35 to 37 weeks is positive for GBS colonization; 2) previous baby born had invasive GBS disease (in this case the mother is not screened for GBS colonization); 3) Positive GBS bacteruria during any trimester of the current pregnancy regardless of the amount of GBS colony forming units per mL of urine (2,71). If the status of maternal GBS colonization is unknown at the onset of the labour then antibiotic prophylaxis is indicated when any of the following risk factors are present: a) delivery at less than 37 weeks of gestation (or preterm labour, in which case the antibiotic prophylaxis is given at the onset of true labour and continued until delivery); b) prolonged rupture for 18 hours or more; c) intrapartum fever is present with temperature equal to or more than 100.4 °F (2,71). The CDC has provided guidelines on how to process the GBS vaginal-rectal swabs in the clinical microbiology laboratory (2): they are inoculated into

the Todd Hewitt broth supplemented with colistin and nalidixic acid. The broth is incubated for 24 hours at 37°C in ambient conditions and sub-cultured to 5% sheep blood agar. The presence of beta haemolytic bacterial colonies is observed on the plates for up to 48 hours. Any beta haemolytic colonies are selected and tested using the catalase test. Colonies testing negative for the catalase enzyme are grouped using the Lancefield grouping kit (identifying which group the Streptococcus belongs to) to confirm the identification of GBS (2). The principle antibiotic that GBS is susceptible to is penicillin. Pencillin G is the preferred treatment and ampicillin can be used as an alternative. If the mother is allergic to penicillin, cefazolin is indicated. If both penicillin allergy and anaphylaxis are present, then clindamycin is indicated (2, 71). Before giving clindamycin, the antibiotic sensitivity test must be performed for both erythromycin and clindamycin. If the isolate is resistant to erythromycin and/or clindamycin, then vancomycin is indicated. As discussed previously, maternal intrapartum antibiotic prophylaxis reduced the rate of EOD, but had no effect on the rate of LOD (Figure 1.1). A surveillance study done in the US monitoring GBS isolates between 1996 and 2005 showed that all isolates were susceptible to penicillin, ampicillin and vancomycin (72); however, some isolates were resistant to erythromycin and clindamycin. A similar study analyzing the antibiotic sensitivity testing on GBS recto-vaginal cultures was performed recently at St. Michael's Hospital in Toronto. Researchers found that all cultures were sensitive to penicillin, 22% were resistant to erythromycin, 19% to clindamycin, and 18% to both erythromycin and clindamycin (73). Although intrapartum antibiotic

prophylaxis is still the preferred treatment for GBS disease, there are problems with antibiotic prophylaxis in the presence of a penicillin allergy, or if anaphylaxis is present. GBS isolates in both Canada and US have been showing increased erythromycin and clindamycin resistance *in vitro*. Therefore, there is a need for a preventative vaccine against GBS infection, especially LOD in neonates.

1.4 Types of Vaccines Developed against GBS.

Many types of conjugated GBS polysaccharide serotype-specific vaccines have been developed against GBS. For example, GBS type III capsular polysaccharide (CPS) - TT (74), GBS type Ia CPS- TT (75) and GBS type V CPS- TT (76) vaccines were tested in non-pregnant women (18-40 years of age). In a separate study, patient sera collected from all three studies were evaluated to check for the duration that the antibodies GBS type III CPS IgG, GBS-type I CPS IgG and GBS-type V CPS IgG were functional in promoting the opsonisation and phagocytosis of GBS specific for the CPS type included in the vaccine (77). All three serotype-specific GBS-CPS IgG had the ability to promote opsonisation and kill only the specific serotypes of GBS cfu/ml by 1 $\log_{10}$ for 18 months to two years post immunization (77). It is clear that vaccines which are suitable against multiple serotypes of GBS and are not based on one specific capsular polysaccharide are needed. One initial strategy used was conjugating the CPSIII vaccine with ScpB (C5a peptidase protein found in multiple GBS serotypes). This vaccine was immunogenic and protective in mice against not only GBS serotype III but also against serotype VI of GBS; the amount of recovered GBS cfu/mg of

mouse lung tissue dropped from $12x10^3$cfu/mg to $1x10^3$cfu/mg post-immunization with CPSIII-ScpB versus CPSIII-TT vaccine alone (78). Protein-based vaccine candidates have also been tested against GBS. Phosphoglycerate kinase (PGK) was recognized as a major GBS outer surface protein using proteomics; newborn mice were then immunized with purified PGK (3). Antiserum that was raised against PGK when added to a plate coated with GBS types I to V showed that PGK was present on the surface of all these serotypes. Additionally, anti-PGK protected neonatal mice against GBS infection; the number of surviving mice increased from 2% to 38% post inoculation with anti-PGK sera mixed with GBS (3). PGK could be developed into a seroprevalent vaccine candidate for GBS. Peptide-based vaccines are another option to explore in the development of GBS vaccines. Developing a peptide-based vaccine is challenging, however, as not all peptides may be immunogenic and some may not recognize the native protein. GBS PGK peptides could be tested as possible vaccine candidates. GBS pili proteins can be used as vaccines to generate immunity in GBS hosts (79). Developing a vaccine that is effective against most serotypes of both Group A *streptococcus* and GBS in neonates could be commercially beneficial. In order to develop a vaccine using GBS-PGK a glycolytic enzyme, it is important to understand the role of PGK in virulence of GBS.

1.5 Streptococcal glycolytic enzymes as a virulence factor

Henderson et al. describe the ability of proteins to have a dual function as protein moonlighting (80). There are many examples of streptococcal glycolytic

enzymes that have functions other than in glycolysis. Some of these surface-expressed glycolytic enzymes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alpha-enolase (α-enolase), and phosphoglycerate kinase (PGK) (5, 81, and 82).

1.5.1 GAPDH and its role in virulence

GAPDH's function is to convert glyceraldehyde-3-phosphate to 1, 3-Bisphosphoglycerate in the glycolytic pathway (Figure 1.5). This enzyme was one of the first to be reported on the surface of GAS (82). This finding led to increased interest in understanding the role(s) of surface-expressed glycolytic enzymes in Gram-positive streptococci. Surface GAS-GAPDH was shown to bind to the eukaryotic proteins fibronectin, lysozyme, and plasminogen; and to the cytoskeleton proteins actin and myosin (82), (83). When surface-expressed glycolytic enzymes bind to eukaryotic proteins, the bacteria may be able disseminate in the host and cause invasive disease. A GAS mutant that lacked GAPDH surface expression did not bind to plasminogen and had only 10% adherence to pharyngeal cells Detroit 562 compared to 30% adherence in the wild type. Additionally, the mutant strain lacked anti-phagocytic activity (84). GAPDH has also been found on the surface of *Streptococcus pneumoniae* and functions as a plasminogen binding protein in this bacterium (7). In GBS, GAPDH was discovered on the bacterial cell surface (36). In this study it was found that, surface-expressed GBS-GAPDH would bind to eukaryotic proteins plasminogen, actin and fibrinogen (36). In a recent study, surface GBS-GAPDH was found to play a role in the apoptosis of murine macrophages (85).

Investigators found that the level of apoptosis decreased when GAPDH was depleted, and they hypothesized that this occurs because inducing apoptosis in the macrophages may help GBS evade the immune system and disseminate further (85). In another study, mice were infected with a GBS mutant that overexpressed GAPDH; post infection, the mice had increased GBS colonization in their livers compared to the mice infected with wild-type GBS (86). When they tested the sera of these mice, there were increased levels of interleukin-10 (IL-10) (immunosuppressive cytokine) present, leading them to conclude that the GAPDH present on the surface of GBS helps the GBS to evade the host immune system by causing immunosuppression in the host(86).

1.5.2 Alpha Enolase and its role in virulence

A second streptococcal glycolytic enzyme is α-enolase. α-enolase is present on the surface of GAS (80, 81), *S.pneumoniae* (6,80) and GBS (3, 80) . Surface α-enolase in both GAS and *S.pneumoniae* has been shown bind to human plasminogen (81, 87) . Surface glycolytic enzymes have been shown to help the bacteria increase its virulence. However, strangely, Mori *et al.* found using a bactericidal assay that in the presence of α-enolase there was increased death of *S. pneumoniae* in human blood, which did not happen when α-enolase was not present (88). Using an *in vitro* migration assay, they also showed that *S.pneumoniae* α-enolase increased neutrophil migrating activity, and induced the formation of neutrophil extracellular traps (NET) (88). They suggest that the NET formation leads to NET-induced neutrophil death (NETosis) helping the bacteria to destroy host tissue (88). Another study found that *S.pneumoniae* α-enolase

may allow the *S.pneumoniae* to evade the host immune system; in this study, the researchers showed that the α-enolase bound to human C4b-binding protein (C4BP) *in vitro,* followed by reduced C3b deposition on the surface of *S.pneumoniae* (87). C4BP is an inhibitor that binds to the C4b component of the human complement pathway, inhibiting the formation of C4b. C4b is part of the C3 convertase, and inhibition of C4b increases the C3 convertase decay. When C3 convertase is absent, the opsonin C3b deposits are reduced, helping the pneumococci evade the human complement pathway (88).

1.6 Phosphoglycerate Kinase: Surface Glycolytic Enzyme of GBS Plays a Role in Virulence

Phosphoglycerate kinase (PGK) is a glycolytic enzyme that is necessary to convert 1, 3 bisphosphoglycerate in the glycolytic pathway (Figure 1.5) (80). PGK is one of *S.pneumoniae's* major surface proteins (89). Surface PGK's role in bacterial virulence has not been studied in great depth in most streptococcal species. In GBS however, PGK was found to be present on the surface and was shown to enhance the virulence of GBS *in vitro* (3, 80) . Understanding the role that surface PGK plays in virulent GBS will help us analyze its potential as a vaccine candidate. In this section I will focus on understanding the expression of GBS-PGK on the surface, and its role in binding host eukaryotic proteins.

1.6.1 Size, location of GBS-PGK

The surface expression of GBS-PGK was first identified when Hughes *et al*. used proteomics to study the various GBS surface proteins (3). The GBS

surface proteins were separated using 2D gel electrophoresis. The peptide sequence of various isolated proteins was converted to nucleic acid sequences (genes) using mass spectroscopy. The nucleic acid sequences (genes) were searched in databases to identify the proteins. One of the proteins identified was PGK. The GBS-PGK nucleotide sequence (3) and a 398-amino-acid-long-protein sequence of surface GBS- PGK (3) were identified. Burnham et al. also identified PGK as being located on the surface of GBS. They described GBS- PGK as an actin-binding protein present on the surface of GBS (90). In the initial stages of investigation, Burham et al. separated the whole GBS lysate and probed with the α-actinin antibody. One of the proteins it bound to was 42 kDa(90). Burham et al. used mass spectrometric analysis followed by BLAST analysis and identified the 42 kDa surface protein to which the α-actinin antibody bound as being PGK from GBS (90) . Both the research studies (3, 90) described the size of surface GBS-PGK as being 42 kDa. As described above, the presence of PGK on the surface is unusual:, ordinarily glycolytic enzymes are located only in the cytoplasm. GBS-PGK is also present on the GBS surface and is secreted into the culture supernatant (5). Boone et al. has found GBS-PGK in the cytoplasm, cell wall, and extracellular region (culture supernatant). The overnight culture of GBS NCS13 was pelleted and supernatant was collected as the extracellular fraction. The resulting pellet was washed and resuspended in the 10 mL spheroplast-forming buffer followed by lysozyme treatment; spheroplast is a cell from which the cell wall is removed. This solution was pelleted and the supernatant was collected as the cell wall fraction. The resulting cell pellet was re-suspended in

distilled water, sonicated and pelleted to collect the cytoplasmic fraction. A second overnight GBS NCS13 culture was resuspended with 100uL of 50mg/ml lysozyme and pelleted to get the whole cell lysate. When Boone et al. probed lysates from these fractions with the anti-GBS-PGK antibody, a 42 kDa size band (PGK) was present in all the fractions (Figure 1.6) (5). Further, GBS cultures probed with anti-GBS-PGK and stained with fluorescent dye showed fluorescence on the periphery of the GBS (5).

1.6.2 Surface expression and secretion of GBS-PGK.

PGK is an anchorless surface protein with no known secretion or surface attachment signals (91). This leads to the question of how GBS-PGK is surface expressed. The hypothesis is that it occurs in two steps: first, it is secreted, and then it attaches to the GBS surface (10). Both the secretion and surface attachment are necessary for surface expression.

1.6.2.1 Attachment of GBS-PGK to the GBS surface

To identify GBS genes involved in GBS-PGK surface expression, Boone et al. performed transposon mutagenesis using the transposon Tn*917*. Tn*917* mutagenesis identified the GBS gene sag1003 was needed for surface expression of GBS-PGK (91).

When transposon Tn*917* was inserted into the *sag1003*, it significantly reduced the binding of GBS-PGK on the GBS surface. The *sag1003* gene is predicted to code for Sag1003 permease protein, which is thought to be an ATP-dependent membrane efflux transporter of peptides in the GBS cell wall (91). A

mutation in the *sag1003* gene may cause the predicted GBS-PGK binding ligand not to be present for the GBS-PGK to bind (91), reducing the GBS-PGK surface expression. The second way in which secreted GBS-PGK attaches to the surface is by interacting with lipoteichoic acid, a major constituent of the GBS surface (10). It was shown that GBS-PGK binds to lipoteichoic acid *in vitro*, and that greater amounts of GBS-PGK bound to the surface at acidic pH levels (10). This mechanism has been shown in *Lactobacillus crispatus:* the GAPDH and Enolase bound to the surface lipoteichoic acids at acidic pH (92). Therefore, Boone et al. suggested in their work that in order for GBS PGK to attach to the GBS surface, there must be a functional *sag1003* gene and lipoteichoic acid interaction (91).

1.6.2.2 Secretion of GBS-PGK

Boone T. further explored the genes involved in PGK secretion (10). He found that the *SecA2* locus and the Srr1 fimbrial protein are partially responsible for secreting GBS-PGK (10). It is important to understand what the *SecA2* locus is and the genes that are located in it. The Sec pathway transports proteins across the bacterial membrane using a Sec protein complex. This complex is in the bacterial cytoplasmic membrane (93). In GBS, the Sec pathway consists of the SecA2-SecY2 complex (93, 94). SecA plays the role of a motor protein (93, 94) , and powers the transport of proteins through the Sec Y channel. GBS has two homologues of SecA protein: SecA1 and SecA2, and an accessory, protein SecY2. The SecA2-SecY2 complex is known to export glycosylated proteins linked to bacterial virulence in *Mycobacteria tuberculosis, Streptococcus gordonii, Streptococcus parasangiunis,* and *Listeria monocytogenes*(93). The

secA2 locus of GBS consists of genes that code for Srr1, GtfA and GtfB, SecA2 and SecY2 proteins (Figure 1.7). Srr1 is a surface glycoprotein (93) that has been shown to function as a fimbrial protein (94) , and contributes to GBS virulence by increasing GBS adherence to host cells (95, 96). The Srr1 protein, together with PiliA protein, has been shown to increase GBS adherence to epithelial cells *in vitro*. Additionally, in the same study it was shown that GBS mutant strains lacking the *srr1* gene colonized the vaginal tract of only 30% of mice, while wild-type GBS colonized 60% mice (95). GtfA and GtfB are glycosylating proteins responsible for the glycosylation and the stabilization (prevention from proteolysis) of Srr1 (94). Therefore, Srr1 cannot function without GtfA, GtfB or SecA2, as it requires GtfA, GtfB for glycosylation and SecA2 for secretion. Previous investigations have shown that to be secreted across the GBS membrane, PGK needs GtfA, GtfB, Srr1 and SecA2 (10). To further understand the role of *secA2* locus proteins in GBS-PGK secretion, Boone used the mutant GBS strains NEM316Δ*secA2* lacking the expression of SecA2, NEM316Δ*srr1* lacking the expression of Srr1, and NEM316ΔgtfA, B lacking the expression of GtfA and GtfB protein (10, 94); these mutant strains were developed by Mistou et al. using the NEM316 GBS serotype III strain obtained from a case of septicaemia (94). The genome of this NEM316 GBS strain has been sequenced (94, 97). The above mutant strains lacking the expression of secA2 protein were found to have less PGK secreted into their culture supernatant than did the wild-type GBS strain (10) . Interestingly, the SecA2 mutant had higher GBS-PGK levels secreted into the culture supernatant than did the wild type (10). Additionally, investigations using

the SecA inhibitor sodium azide showed that SecA inhibition prevented PGK secretion into the supernatant (10). This suggested that SecA somehow also plays a role in GBS-PGK secretion (10). The mutant NEM316 GBS strains NEM316Δ*secA2*, NEM316Δ*srr1*, and NEM316Δ*gtfA, B* had lower levels of PGK expression than did the wild-type GBS strain, suggesting that Srr1, GtfA and GtfB play a role both in secreting and attaching PGK to the GBS surface. Furthermore, it was shown that lower quantities of exogenously added GBS-PGK bound to the above GBS mutant strains that were lacking the expression of the Srr1, GtfA and GtfB proteins. The SecA2 based secretion of glycolytic enzymes has been demonstrated in another Gram positive bacterium, *L. monocytogenes* (98). The inhibition of the *secA2* gene inhibited the secretion of α-enolase in *L. monocytogenes* (98).

1.6.3 Binding Plasminogen to activate plasmin: A virulence factor in bacteria

As reported in the previous sections, many bacteria use surface glycolytic enzymes as a virulence strategy. Bacteria secrete glycolytic enzymes that attach to host proteins. This helps the bacteria to invade host cell tissue barriers and avoid the host immune attack. Glycolytic enzymes such as α-enolase, GAPDH and PGK have been shown to bind host eukaryotic proteins and increase bacterial virulence. For example, GAS mutant lacking GAPDH was deficient in plasminogen binding and had no anti- phagocytic activity (i.e., it couldn't evade the host immune system) (84). GBS-PGK has been shown to bind the eukaryotic proteins plasminogen, actin, fibrinogen and fibrin in vitro (84, 91). The purpose

of this section is to understand plasminogen binding of GBS-PGK and how it can contribute to virulence.

Plasminogen binding is the focus of this section because it is seen in many bacteria (4-8) and fungi (9) as a key factor that helps to invade the host. Human plasminogen is a 92 kDa protein that circulates in the human plasma. Plasminogen in humans is normally activated by two plasminogen activators: the urokinase plasminogen activator (upA) and tissue-type plasminogen activator (tpA)(8). The activated form of plasminogen is a serine-rich protease plasmin. There are two ways for bacterial activation of plasminogen to occur (Figure 1.8). The first is that the bacteria possess plasminogen receptor molecules which bind to plasminogen and recruit it to the bacterial surface (99) (Figure 1.8). Host plasminogen activators tpA or upA then activate the surface-bound plasminogen to plasmin. Bacteria use this strategy to break down the extracellular matrix proteins fibronectin and laminin (8, 99) . An example of this strategy is the GBS Skizzle protein (100). The Skizzle protein was shown to bind plasminogen and then activate to plasmin by human upA and tpA (100). GAPDH binds to plasminogen; and the GAPDH-plasminogen complex activated plasmin using tpA and upA, which promoted invasion of GBS as shown in a mouse model (101). The second way is for bacterial plasminogen to bind and activate directly to plasmin (Figure 1.8) (99). An example of this strategy is the streptokinase-plasminogen binding protein released by groups A, C and G streptococci that binds plasminogen to form a complex and activates it to plasmin (8). This strategy helps the bacteria to break down fibrin clots formed as a result of

inflammation and invade further into the host cells (99). Therefore, the binding of glycolytic enzymes to plasminogen and activation to plasmin is a very important virulence factor and has been studied extensively.

1.7 Rationale for the Proposed Research

Current research has identified three proteins on the surface of GBS that bind plasminogen: GAPDH (101), Skizzle (100) and PGK (5) . PGK on the surface of GBS is thought to act like a plasminogen receptor, and has been shown to bind plasminogen *in vitro* (5). The major plasminogen binding domains of GBS-PGK have been located between amino acids 126-130, 154-165, 202-205, 207-221, and 291-305 of the GBS PGK molecule, a chain that contains 398 amino acids (4). To better understand the *in vivo* virulence of GBS-PGK binding to plasminogen; it is necessary to study a GBS strain lacking PGK binding to plasminogen. Since there is only one copy of the PGK gene in the GBS genome, it is not possible to perform a knock-out mutation to make a mutant GBS-PGK molecule that does not bind to plasminogen (4, 102) . Boone et al. used site-directed mutagenesis to create a GBS-PGK mutant that exhibited a 23% reduction in binding to plasminogen while retaining its glycolytic activity (4, 10). This mutant PGK-M8 was created through lysine substitutions to alanines at amino acids 302 and 306, which reduced the binding to plasminogen in vitro (4) . Lysine residues are involved in binding PGK to plasminogen; previously, it was shown that incubating rGBS-PGK with lysine analogue 6-aminocaproic acid (6-ACA) reduced the binding of rGBS-PGK to plasminogen *in vitro* (5, 10). Lysine residues from streptokinase (8) and Skizzle protein (100)

have also been shown to be involved in plasminogen binding. While the above lysine to alanine substitutions in PGK-M8 resulted in lower levels of plasminogen binding, they did not completely abolish it. The purpose of my research was to create a GBS-PGK protein that retained its glycolytic activity yet did not bind to plasminogen. This thesis used PGK-M8 (10), the previously created GBS-PGK mutant molecule containing substitutions converting lysine to alanine at 302 and 306. I chose this mutant as it had significantly reduced binding to plasminogen and was glycolytically active. In addition, the lysine at 302 and 306 was found to localize near glutamic acid residue 309. Glutamic acid residues are negatively charged, and negatively charged residues have been shown to be needed for plasminogen binding. The requirement for negatively charged residue aspartic acid in plasminogen binding sites has been demonstrated for *S. pneumoniae* α-enolase (103). Substituting aspartic acid to alanine in the internal plasminogen binding site (with amino acid sequence FYDKERKVYD) of *S.pneumoniae* α-enolase was found to abolish binding to plasminogen *in vitro* (103). Accordingly, substitution of glutamic acid at position 309 to alanine in the PGK-M8 mutant may abolish plasminogen binding in surface GBS-PGK without affecting glycolytic activity.

1.8 Hypothesis and Objectives

My Hypothesis:

Triple alanine substitution at positions 302^{lys}, 306^{lys} and 309^{glu} in GBS-PGK retains glycolytic activity but abolishes binding to plasminogen when compared to double alanine mutant PGK 302^{lys} 306^{lys} or wild type GBS-PGK.

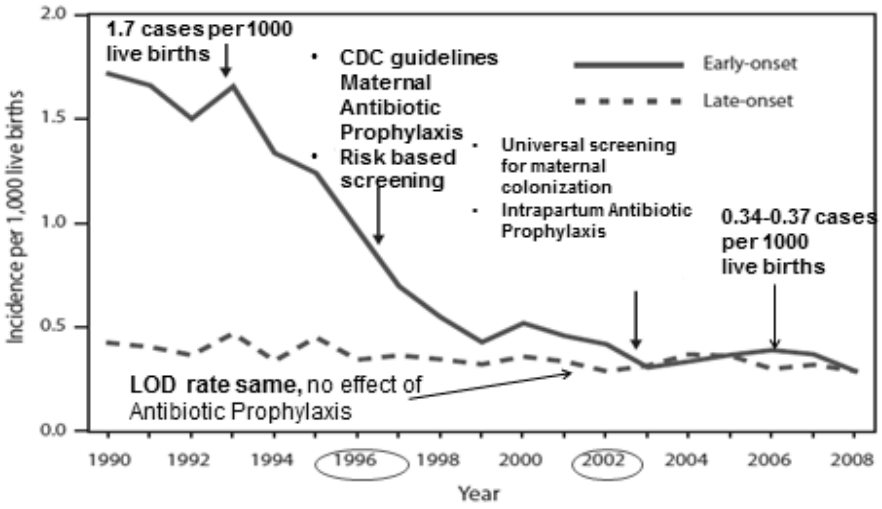
The objectives of this thesis are:

1. To create the PGK-M9 triple mutant from PGK-M8 double mutant using site-directed mutagenesis to substitute glutamic acid at position 309 with alanine.

2. To determine the glycolytic activity of the triple mutant PGK-M9 and compare it with glycolytic activity of double mutant PGK-M9 and wild type PGK using an enzyme assay for PGK.

3. To determine the plasminogen binding ability of triple mutant PGK-M9 using ELISA assays compared with double mutant PGK-M8 and wild type PGK.

Figure 1.1: Incidence of Group B Streptococcal disease in North America 1990-2008



In the early 1990's the rate of early onset GBS disease was 1.7 cases per 1000 live births. In 1996, the CDC implemented guidelines for maternal GBS screening and maternal antibiotic prophylaxis, which led to a decrease of EOD in neonates. The rate of GBS disease decreased to 0.34-0.37 cases per 1000 live births in the late 2000's. The rate of late onset disease (LOD) remained same, at 0.4 cases per 1000 live births, from the 1990s to 2000's. This image was reproduced with permission and adapted from Verani *et al*., 2010 (2).

Table 1.1: Rates of invasive neonatal GBS disease in Alberta (2010 and 2011)

	Rate/1000 live births (number of cases)	
Year	Early onset disease	Late onset disease
2010	0.34 (17)	0.26 (13)
2011	0.28 (15)	0.30 (16)

Early onset disease (EOD) is classified as occurring at between 24 hours and seven days of age. LOD is classified as occurring beyond seven days and up to three months of age**.**

Table 1.2: GBS surface binding proteins

GBS Surface Protein	Host cell Protein binding
PilA, PilB	von Willebrand
FbsA, FbsB	Fibrinogen
ScpB	Fibronectin , Integrins
αCP	GAG, integrins
GAPDH	Plasminogen
PGK	Plasminogen

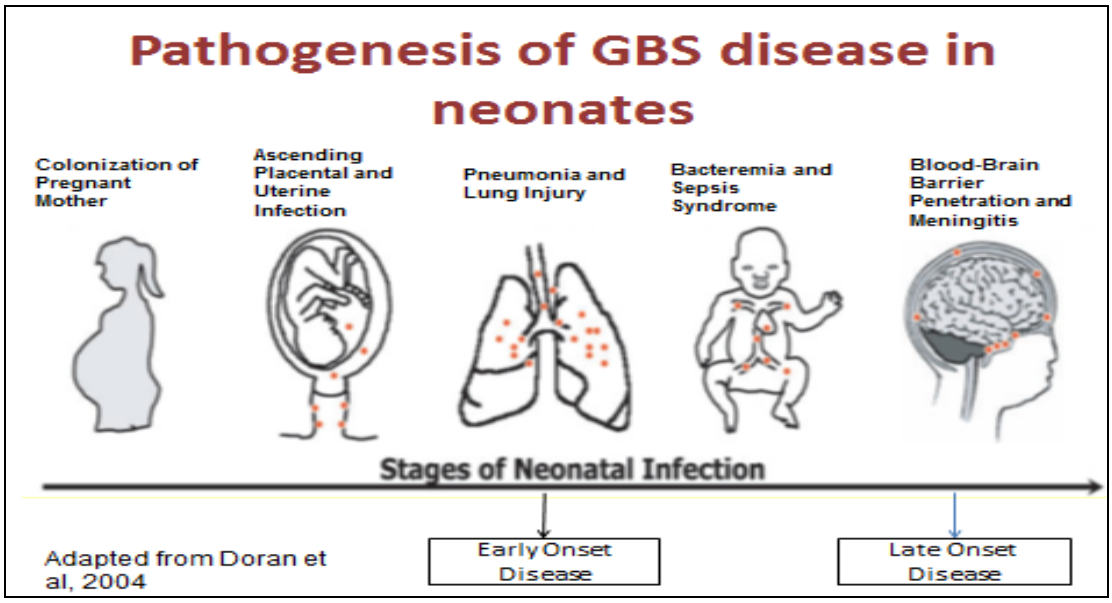


Figure 1.3: Stages of GBS neonatal infection

From a disease progression perspective, in summary (Figure 1.3), GBS disease in neonates begins with 1) the GBS ascending from the vaginal area of a colonized pregnant mother and 2) crossing the placental membranes and causing *in-utero* infection. 3) The neonate breathes in the GBS during the birth process or *in utero,* causing pneumonia. 4) The GBS is able to invade further into the bloodstream, causing septicaemia. 5) GBS can also invade the neonatal blood-brain barrier, causing meningitis (1). EOD is typically expressed as pneumonia which may progress to sepsis. LOD can be expressed as a bacteremia, but the majority of cases present as neonatal meningitis. This image was reproduced and adapted with permission from Doran *et al*., 2004 (1).

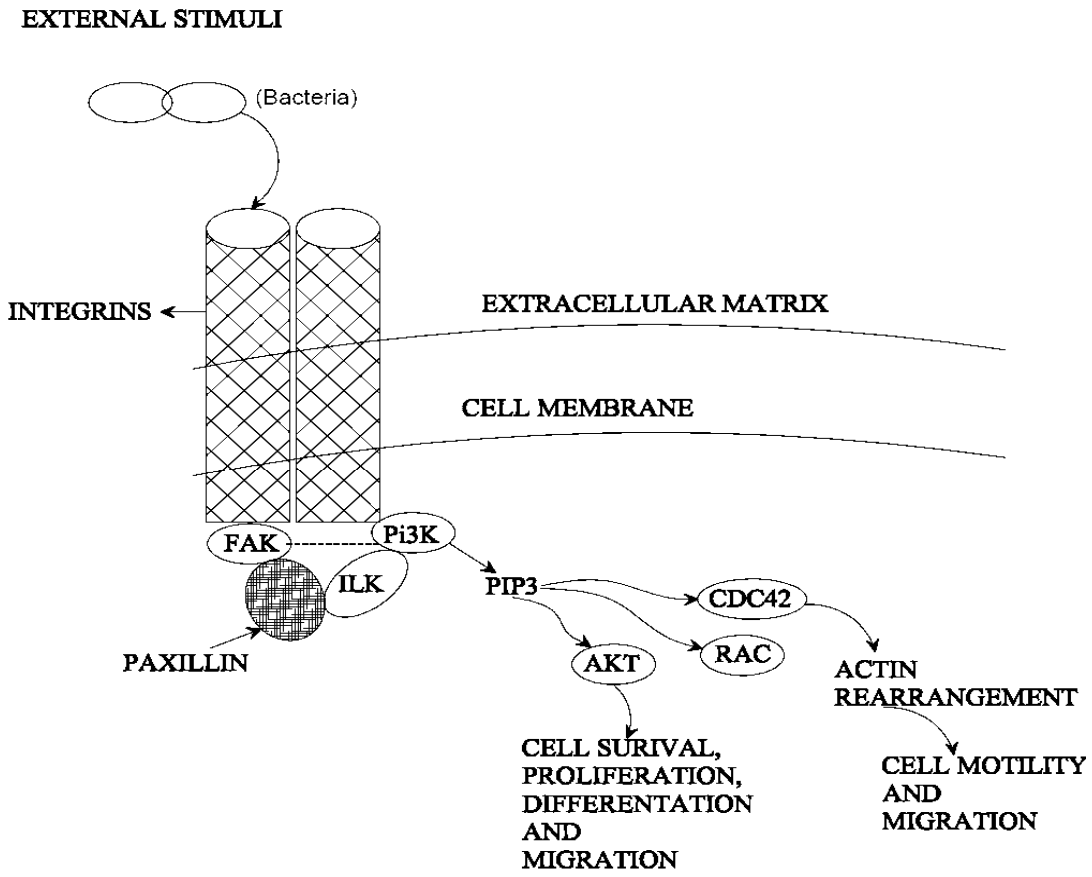


Figure 1.4: Possible pathway of GBS invasion of host epithelial cells

GBS αCP binds to the epithelial cell via the α1β1 integrin. FAK/ILK is phosphorylated, causing the paxillin adaptor protein to attach, forming an adhesion complex which binds integrin and focal adhesions are activated. FAK/ILK interacts with the PI3K and activates the PI3K/AKT pathway and/or the PI3K activates Rac 1. Both these pathways can cause actin polymerization and actin cytoskeleton motility.

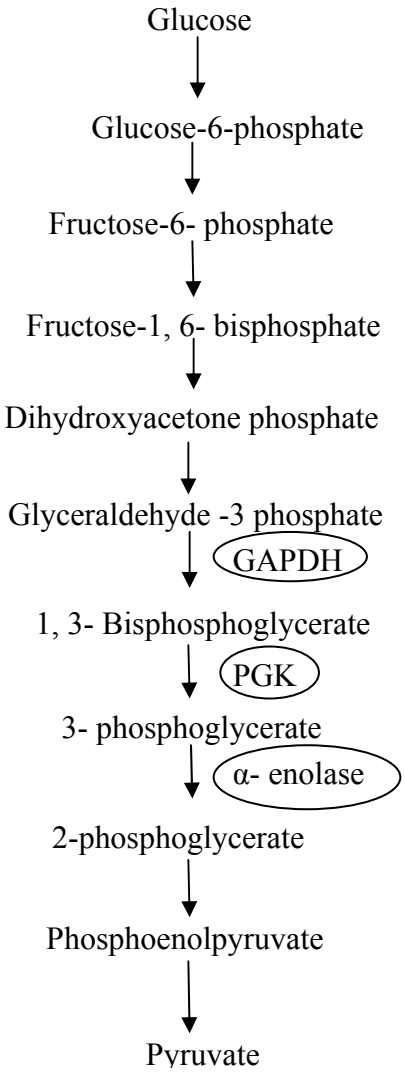


Figure 1.5: The streptococcal glycolytic enzymes involved in the glycolytic pathway

This figure of the glycolytic pathway highlights glycolytic enzymes that are also expressed on the bacterial surface: GAPD, PGK and α-enolase.

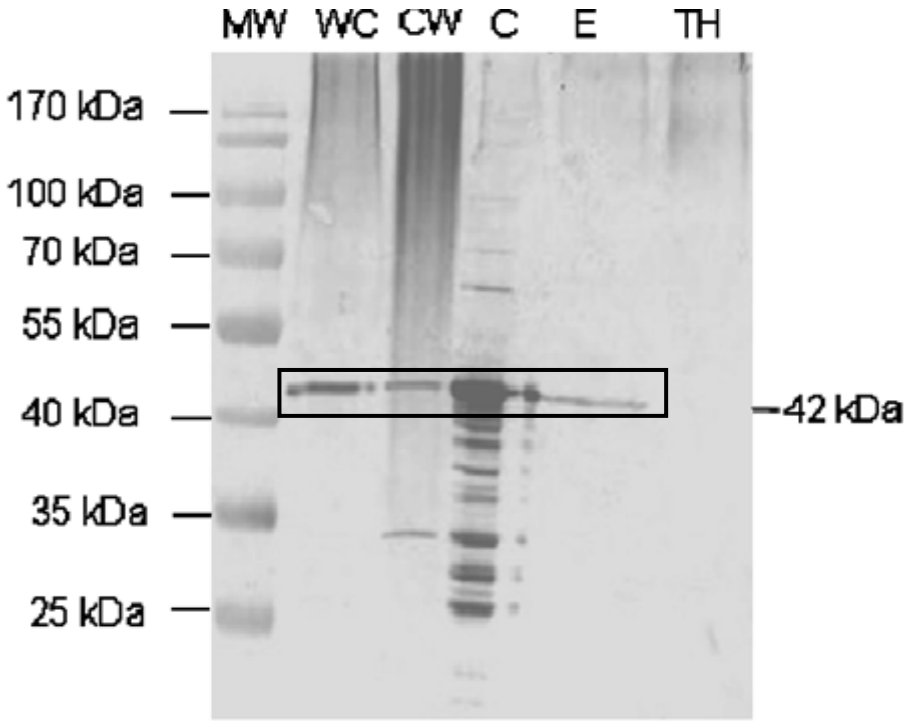
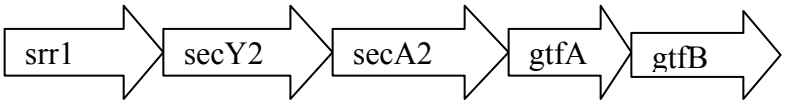


Figure 1.6: Western-blot fractions of GBS lysates

Whole cell lysates and cell fractions from GBS were probed with anti-GBS-PGK antibody. A 42 kDa-size band (PGK) was present in all the fractions (5). This image was reproduced with permission and adapted from Boone et al. 2011 (5).

MW = Molecular weight, WC= Whole Cell lysate, CW= Cell wall fraction, C= Cytoplasmic fraction, E= Extracellular fraction, TH= Todd Hewitt.



srr1 gene codes for Srr1 surface glycoprotein that functions as a fimbrial protein

secY2 gene codes for SecY2 accessory protein

secA2 gene codes for SecA2 motor protein

gtfA and gtfB genes code for GtfA, GtfB glycosylating protein respectively.

Figure 1.7: SecA2-SecY2 locus of NEM316 GBS strain

The NEM316 GBS serotype III strain was obtained from a case of septicaemia. The genome of this NEM316 GBS strain has been sequenced and was used to understand the function of secA2 locus proteins (94, 97). The *secA2* locus of GBS consists of genes coding for Srr1, GtfA and GtfB, SecA2 and SecY2. Boone et al. explored the genes involved in PGK secretion. He found that *SecA2* locus and the Srr1 fimbrial protein are partially responsible for secreting GBS-PGK (10) .

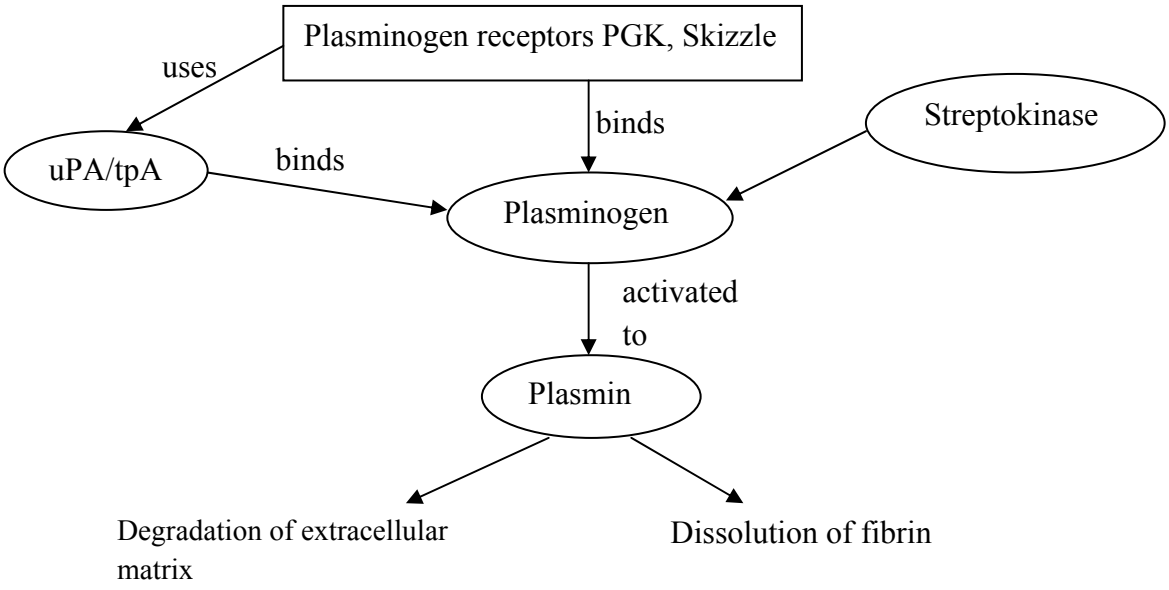


Figure 1.8: A model pathway depicting two ways by which streptococcal bacteria may activate plasminogen

There are two ways for bacterial activation of plasminogen. The first way involves the bacteria to recruit its plasminogen receptor molecules to the bacterial surface (99). An example of this strategy is the GBS Skizzle protein (100). The second way involves the bacteria directly bind and activate plasminogen (99). An example of this strategy is the streptokinase plasminogen binding protein released by groups A, C and G streptococci that binds plasminogen to form a complex and activates it to plasmin (8).

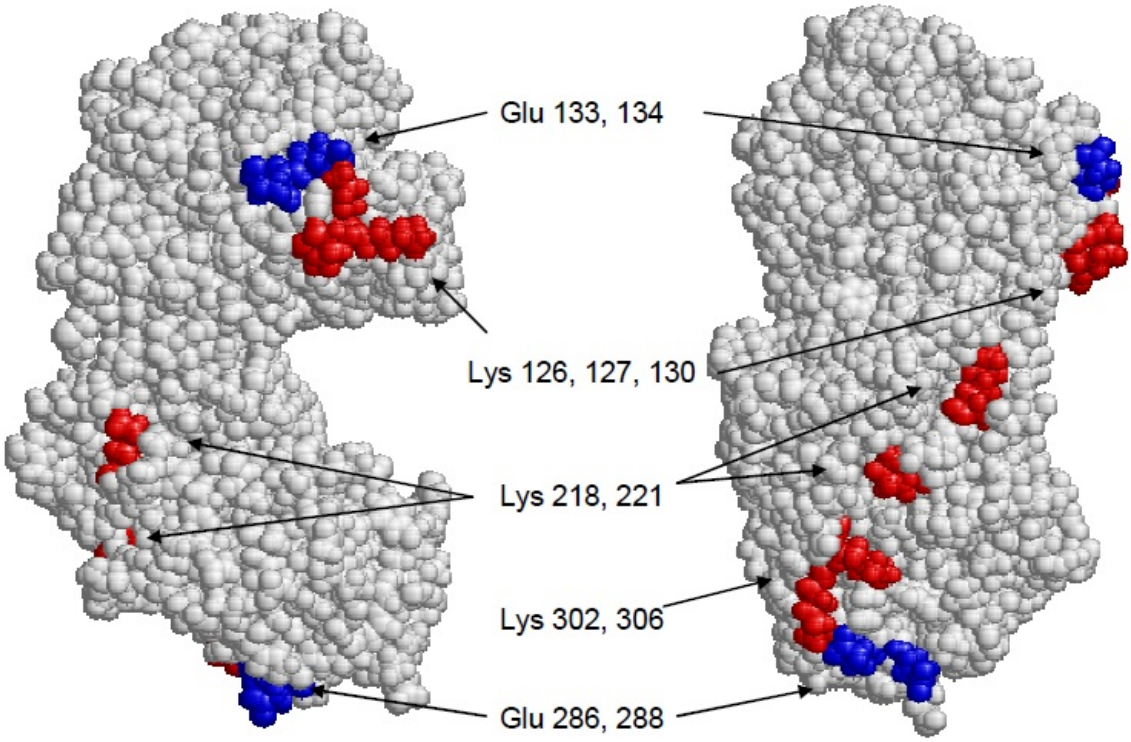


Figure 1.9: Space-filling models of GBS-PGK. Actin and plasminogen binding regions of GBS-PGK

Boone et al. (2012) submitted the amino acid sequence of GBS-PGK to iTASSER (4) to generate a model GBS-PGK structure. They used RasMol to visualize the model structure (4). The image above highlights the lysine (red) and glutamic acid residues (blue) involved in actin and plasminogen binding. These residues were identified using site-directed mutagenesis. This image was adapted with permission from Boone et al. (4).

Chapter 2:

Materials and Methods

2.1 Bacterial strains and growth conditions

The *Escherichia coli* (*E. coli*) strains used in this research study are described in Table 2.1. *E.coli* from frozen laboratory stocks was grown in Luria-Bertani (LB) broth or on LB agar at 35°C (unless other conditions are described). Antibiotics (25 μg/ml kanamycin, 60 μg/ml carbenicillin) were added when required.

2.2 Site-directed mutagenesis

The *pgk* gene from the PGK-M8 mutant, which contains substitutions converting lysine 302, 306 to alanines, was subjected to site-directed mutagenesis following the procedure provided in the GeneTailor Site-Directed Mutagenesis system (Invitrogen; Carlsbad, CA, USA). The plasmid DNA was first methylated for one hour in a 37°C water bath. The methylation reaction was done using the DNA methylase enzyme to methylate the cytosine residues of the double stranded DNA. The methylated DNA was amplified using PCR primers described in Table 2.2, with the PGK M7oL reverse primer containing the target mutation. A PCR reaction of 50 μl was set up using the components described in Table 2.2. The PCR amplification included the following cycles: one cycle of 94°C for two minutes, followed by 20 cycles each of 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 4 minutes (the PGK sequence is about 4 kb; 1 min/1 kb DNA) and ending with one cycle of 68°C for 10 minutes. After site-directed mutagenesis, the *pgk*-M8 sequence was linearized with one single nucleotide changed. The DNA was run on a 1% agarose gel to analyze the efficiency of mutagenesis. High efficiency mutagenesis should not have smeared bands, according the GeneTailor

Site-Directed Mutagenesis system instruction manual. To remove the methylated plasmid and circularize the mutated DNA, the linear DNA mutagenesis product was transformed into One-Shot MAX Efficiency DH5α-T1 *E. coli* competent cells (Invitrogen ,Carlsbad, CA, USA). The *Mcr*BC endonucleases of the *E.coli* digest the methylated template and leave the un-methylated circularized plasmid DNA with the mutation. Mutagenesis resulted in an adenine-to-cytosine mutation at position 926 of the *pgk* gene, which led to a change of codon GAA to GCA in the *pgk gene*. The transformed mutated plasmid was extracted from the competent cells using the Qiaspin Mini Prep kit (Qiagen; Mississauga, ON, CA).

2.3 DNA sequence of *pgk*-M9 gene

The *pgk*-M9 gene was DNA sequenced to confirm the presence of the adenine-to-cytosine mutation and the presence of previous pgk-M8 mutations. Later, the PGK-M9 sequence was aligned with the GBS-PGK sequence (Table 2.3). The alignment helped determine if the pgk-M9 sequence was correct, and displayed the required changes of AAA to GCA 302, AAA to GCA 306, and GAA to GCA 309. A space-filling protein structure model of PGK-M9 highlighting the location of the three alanine substitutions was visualized using the RasMol protein structure software. RasMol software that allows viewing and analyzing the structure of biological proteins was used.

2.4 Cloning the *pgk*-M9 into pQE30

The *pgk-M9* was cloned into pQE30 expression vector (Figure 2.2) using the QIAexpressionist kit (Qiagen; Mississauga, ON, CA). The transformed

mutated plasmid was extracted from the *E. coli* DH5α competent cells using Qiaspin Mini Prep kit (Qiagen; Mississauga, ON, CA). The *pgk*-M9 (Insert) gene was PCR amplified using the primers PGK-*Pst* primer (reverse) 5`TTCCTGCAGTTATTTTTCAGTCAATGC3` and PGK-*Bam* primer (forward) 5`TTCGGATCCGCTAAATTGACTGTTAAA3` (Figure 2.2). The added restriction sites were used to ligate the *pgk*-M9 fragment to the pQE30 vector. The amplified *pgk*-M9 gene was purified using the PCR purification kit (Qiagen; Mississauga, ON, CA). Five µL of the PCR reaction was electrophoresed on a 1% agarose gel to check for purity of the reaction and check if the amplicon was the correct size (Figure 2.3). The insert pgk-M9 (1 µg) was restricted with restriction enzymes *Pst* I and B*amH*I. The amount of *pgk*-M9 PCR amplicon present was measured using a NanoDrop (Thermo Scientific) absorbance spectrophotometer. This analysis also helps analyze the purity of the DNA. The DNA absorbance ratio (260/280) at 260 nm and 280 nm is used to assess the purity of the DNA and RNA. A ratio of 1.8 is acceptable for "pure" DNA. One microgram of this insert was restricted using 1 µL each of the restriction enzymes *Pst* I and *Bam* HI and 2.5 µL of Buffer K (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 1000 mM KCl) (Invitrogen, Carlsbad, CA, USA) to a total volume of 25 µL. The restriction digestion was incubated at 37^0C for 1 hour. The pQE30 vector was extracted from the JM101 *E.coli* frozen laboratory stocks using the Qiaspin Mini Prep kit (Qiagen; Mississauga, ON, CA). One µg of the vector pQE30 was restricted with *Pst*I and *Bam*HI for 1 hour at 37^0C in a restriction digest similar to the insert.

2.4.1 Ligation of pgk-M9 into pQE30

The vector and insert (ratio 2:1), ligase buffer (Invitrogen, Carlsbad, CA, USA), and the T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) were added to a total volume of 20 µL. The ligation reaction was carried out overnight at room temperature. The normally recommended ratio of vector: insert is 1:3. However, in figure 2.3, the amount of pQE30 is lower than the insert. When a 1:3 ratio of vector: insert was used in the ligation reaction, the ligated plasmid when transformed did not grow any colonies on the selective plate. Vector ends of 30 fmol and insert ends of 90 fmol are sufficient for the ligation reaction to work. The following calculation was used to approximate the volume of insert and vector needed to get 30fmol and 90fmol respectively.

- The ng of DNA present

  = fmole DNA x1 µg/3000 fmol x size of DNA bp/1000 bp

  = 90 fmol DNA x 1 µg/3000 fmol x 1200/1000bp

  = 36 ng of insert amount will make 90 fmol. Similarly, 33.6 ng of vector will make 30 fmole.

  In this research study,

  Size of insert = 1200 bp

  Size of vector = 3461 bp

  The fmol of Insert = 90

  fmol of Vector = 30

- Volume of Vector and Insert needed

  = ng of Vector or Insert/concentration of Vector or Insert* (ng/µL)

= 36ng of Insert in 90 fmol/30.48 ng/µL and 33.63 ng of Vector in 30 fmol/18.81 ng/µL

= approximately 1 µL of Insert and 2 µL of Vector.

The Vector and Insert were purified to remove the restriction enzymes and measured on a NanoDrop spectrophotometer. The concentration of Vector was 18.81ng/µL and Insert was 30.48 ng / µL.

2.4.2 Transformation of ligated construct into M15 pREP4 E.coli cells

The ligation mix was transformed into chemically competent ($CaCl_2$) M15 pREP4 *E.coli* cells. The pREP4 plasmid of M15 pREP4 *E.coli* expresses a lac repressor gene called *lac I* gene which controls the lac operon of pQE30 from expressing proteins unless induced. The lac operon suppression is necessary for efficient expression of *pgk*-M9 gene. If expression is not supressed, then the lac operon may cause the production of leaky proteins, which may be toxic to the host. This will lead to reduced expression of recombinant protein. A single colony of M15 pREP4 *E.coli* was grown in 100 mL LB broth with 25 µg/mL of kanamycin for 3 hours at 35^0C. The *E.coli* cells were treated with 0.1M $CaCl_2$ and stored on ice to make them chemically competent. Chemically competent cells will have the positively charged Ca^{2+} ion which will attract the negatively charged plasmid DNA, making the uptake of the foreign DNA more favourable. In the next step, the ligated vector and insert mixture and the competent cells were incubated on ice for 30 minutes, allowing for the ligated plasmid to come into

contact with the competent cells. The transformation mix of competent cells and plasmid DNA was heat shocked at 42^0C for 90 seconds followed by rapid cooling in an ice bath (0^0C). Once the ligated mixture was transformed, the nutrient-rich SOC (super-optimal broth, $MgCl_2$ and sucrose) medium was added. The mix was incubated for 45 minutes at 35^0C with rapid shaking. LB plates containing both kanamycin (25µg/mL) and carbenicillin (60 µg/mL) were warmed to 35^0C. The transformed cells were plated out using a spreader and incubated overnight at 35^0C for 16 hours. The key things to monitor in the transformation procedure were 1) good competent cells 2) optimal incubation of DNA with competent cells on ice 3) accurate temperature and time of heat-shock followed by rapid cooling 4) recovery of bacteria in super optimal broth with glucose (SOC) and expression of antibiotic resistance.

2.4.3 Controls for Ligation and Transformation

To assay for the efficiency of the pQE30 restriction, the first control was a *BamH*I-*Pst*I restricted pQE30 only ligation reaction with no *pgk*-M9 insert. The transformation of the pQE30 only ligation control grew very few colonies compared to the actual transformation. This indicated that the vector pQE30 was restricted properly by the restriction enzymes *Bam* HI and *Pst* I, as very few colonies were present in the actual transformation (104).

During transformation, unrestricted pQE30 was added to competent cells to assay for any problems with the competent cells, transformation procedures or antibiotic selection (104). Many colonies grew, indicating that the transformation was efficient. Before protein expression, clones were assayed for the presence of

the desired construct. Each individual transformed colony was grown in LB broth with kanamycin (25 µg/ml) and carbenicillin (60 µg/mL). The plasmid from the clones was extracted using the Qiaspin mini prep kit and restricted with *Pst* I and *Bam* HI and electrophoresed on a 1% agarose gel to assay for the construct PGK-M9.

2.5 PGK-M9 protein expression and purification

The PGK-M9 protein was expressed using the Qiaexpressionist kit (Qiagen; Mississauga, ON, CA) and procedures described by Boone et al. (10). PGK-M8 and the wild-type construct rGBS-PGK (5) from frozen laboratory stocks were also purified and expressed using the Qiaexpressionist kit. The pQE30 vector used in the PGK-M9 clone allows the PGK-M9 protein to be expressed and purified as an N-terminal hexahistidyl-tagged (6xHis-tag) protein. Rapid screening of small expression cultures was done to check if the clones expressed the recombinant protein PGK-M9. Clones expressing the recombinant protein (Figure 2.4) were grown overnight in 1.5 ml LB broth supplemented with 25µg/mL kanamycin and (60µg/ml) carbenicillin. Pre-warmed 10mL LB broth with kanamycin (25µg/mL) and carbenicillin (60µg/mL) was inoculated with the overnight culture of clones expressing PGK-M9 and grown at 35^0C with vigorous shaking. The OD_{600} value was measured every 30 minutes using an absorbance spectrophotometer until an OD_{600} value of 0.60 was obtained (approximately one hour). An OD_{600} value of 0.5-0.7 for *E. coli* was ideal for PGK-M9 protein expression. To obtain the good yield of recombinant protein, the *E. coli* was induced during growth phase. Once the growth phase is passed, the *E. coli* will

start producing toxic proteins leading to a very low yield of recombinant protein. The PGK-M9 protein expression is induced by adding 2mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The IPTG binds to the Lac repressor protein encoded by the *lac* I gene of plasmid pREP4 and inactivates the repressor protein. In the pQE30 vector (Figure 2.5), the pgk-M9 gene is cloned into the *BamH*I, *a PstI* cloning site located downstream of the two Lac-operons and the T5 promoter. The two lac-operators in the pQE30 vector (Figure 2.5), together with the lac repressor protein from pREP4, allow for tight regulation of protein expression (The QIAexpressionist, 2003). The lac repressor protein stays bound to the lac-operator until the IPTG inducer is added, which prevents the production of leaky proteins. The added IPTG binds to the lac repressor protein, inactivates it and stays bound to the repressor, as it cannot be catabolized by the bacteria. Once the lac repressor is inactivated, the transcription can begin downstream from the T5 promoter. The transcripts produced are then translated into the recombinant PGK-M9 protein. The induction and protein expression was carried out for four hours at 35^0C with rapid shaking. Before adding the IPTG (T0) and at 4 hours after induction (T4), an aliquot was assayed on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.2). The rapid small-scale expression helped to analyze which of the clones had optimal protein expression and determine the right temperature and time for protein expression.

2.5.1 Protein purification

Protein purification (Figure 2.6) of PGK-M9, PGK-M8 and rGBS-PGK was carried out under native conditions using the Qiaexpressionist kit (Qiagen;

Mississauga, ON, CA) and methods described by Boone et al. (5). The transformed clones with the greatest protein expression from the small-scale protein expression assays were grown overnight in LB supplemented with kanamycin and carbenicillin. Ten millilitres of overnight culture were used to inoculate 100 ml of LB broth with (25µg/ml) kanamycin and (60µg/ml) carbenicillin. When the OD_{600} value was 0.6, the expression was induced by adding IPTG (2mM). This was then incubated for four hours at 35°C; after which the bacterial cells were harvested by centrifugation at 5000 x g for 20 minutes. The bacterial cell pellet was frozen overnight at -70^{0}C, thawed on ice for 15 minutes and weighed. It was re-suspended in 2ml of lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole and pH adjusted to 8.0 using NaOH) per gram of pellet weight and treated with 1mg/ml of lysozyme for 30 minutes on ice. For example, a 2.86 grams bacterial pellet was re-suspended in 5.72 ml of lysis buffer. Sonication in an ice water bath for 15 minutes was done to lyse the bacterial cells completely. Following lysis, the cells were centrifuged at 10,000 x g for 30 minutes at 4^{0}C to pellet the cellular debris. The supernatant (lysate) resulting from cell lysis was decanted and incubated with 1ml 50% Ni-NTA slurry (Qiagen; Mississauga, ON, CA) for 1 hour at 4^{0}C with shaking. The lysate-Ni-NTA mixture was loaded into a 5ml polypropylene column (Qiagen; Mississauga, ON, CA) and washed with 8 x 1ml of wash buffer (50 mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0). Purified PGK-M9, PGK-M8 and rGBS-PGK proteins were eluted 6 times with 500 µl aliquots of elution buffer (50mM Na2H2PO4, 300mM NaCl, 250 mM imidazole, at pH 8.0). The entire

lysis procedure was carried out on ice or at 4^0C to prevent protein degradation. Since this purification was performed under native conditions, a low concentration of imidazole was added to the lysis and wash buffers to minimize binding of untagged contaminating proteins.

2.5.2 SDS-PAGE

Purified proteins were separated by 10% SDS-PAGE gel to determine the size of the protein and check that the protein was sufficiently purified and was free of any other contaminating proteins. The SDS in the SDS page is a dissociating agent that is negatively charged (cationic). Adding an excess of SDS makes the intrinsic charge of the proteins insignificant compared to the negative charges provided by the SDS. This makes the protein migration based on size and not charge (105). The gel was made by pouring the 10% resolving gel (40% Acrylamide-bisacrylamide, 1.5M Tris-HCl pH 8.8, SDS,APS, and TEMED) followed by a 4% stacking gel (same components as 10% gel, except contains 0.5M Tris-HCl at pH 6.8). The acrylamide polymerizes and the bisacrylamide cross-links to form the gel. This process of gel formation is initiated by ammonium persulphate (APS) and the catalyst TEMED. In the APS-TEMED system, the TEMED catalyzes the formation of free radicals from persulphate ions and these in turn initiate acrylamide polymerization. Therefore, it is imperative for optimal polymerization to de-gas the poured gel by adding ethanol on the top. The sample buffer, which is used to dilute the samples, contains glycerol, SDS and mercaptoethanol. The glycerol increases the density of the protein sample so that when applied to the wells of the gel, the sample remains in the defined well

and does not overflow and mix. The mercaptoethanol disrupts the protein polypeptide disulphide bonds. Purified proteins diluted in 5x sample buffer were loaded into the two-part gel containing 4% stacking gel and 10% resolving gel. The proteins were separated at 120V for 90 minutes by adding 1x running buffer containing glycine. Once the gel was completed, it was stained with Coomassie Blue stain for one hour followed by de-staining with ethanol until bands were visible.

2.6 Glycolytic enzyme activity of PGK-M9 protein

The glycolytic activity of purified PGK-M9, PGK-M8 and rGBS-PGK was measured as previously described (106, 10). The principal behind the assay is to determine the glycolytic activity by measuring the production of NADH at 25°C and at pH 6.7 based on the following coupled reaction:

$$\text{GAP}+\beta\text{-NAD}+\text{Pi} \xrightarrow{\text{GAPDH}} \text{1, 3- Bisphosphoglycerate} + \beta\text{-NADH}$$

$$\text{1,3-Bisphosphoglyerate} + \text{ADP} \xrightarrow{\text{PGK}} \text{3-Phosphoglyerate} + \text{ATP (106)}$$

The change in absorbance at 340nm as the result of the production of NADH in the presence of rGBS-PGK, PGK-M8 and PGK-M9 was measured. The following formula was used:

$$\text{Units/mg enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta_{340nm}/\text{min Blank})(3)}{(6.22 \times [\text{PGK}])}$$

3 = Total volume of reaction mix (milliliters)

6.22 = millimolar extinction coefficient of β-NADH at 340 nm.

[PGK]= amount of rGBS-PGK, PGK-M9 or PGK-M8 used in milligrams

Blank= 100mM postassium phosphate

Positive Control= PGK derived from *Saccharomyces cerevisiae*

- According to the equation and formula given above, one unit can be defined as one milligram of PGK enzyme required to convert 1µM of 1, 3-Bisphosphoglyerate to 3-Phosphoglyerate per minute at pH 6.7 and 25^0C (pH and temperature of reaction mix).

The glycolytic enzyme assay was carried out by adding 2.4µg PGK-M9, rGBS-PGK or PGK-M9 to a 3mL reaction mix. Reaction mix was equilibrated before addition of 2.4ug of PGK-M9 or PGK-M8 or rGBS-PGK. The reaction mix contained 50mM potassium phosphate, 0.83mM glyceraldehyde-3-phosphate, 0.3 mM β-nicotinamide adenine dinucleotide, 0.2mM adenosine 5' –diphosphate, 4.2 mM magnesium sulphate, 133 mM glycine and, just prior to measurement, 1 unit (10µl of 20µl/ml) of glyceraldehyde-3-phosphate (4). The absorbance at 340 nm was measured using an absorbance spectrophotometer for five minutes and the change in absorbance was used to calculate the enzymatic activity. The glycolytic activity of PGK-M9 was compared to the glycolytic activity of a positive control of 0.06 units (U) PGK (Sigma Aldrich, St. Louis, MO, USA) purified from *Saccharomyces cerevisiae* and to the glycolytic activity of purified PGK-M8 and rGBS-PGK. A negative control potassium phosphate (100mM) was used. The purified glycolytically active PGK-M9, PGK-M8 and rGBS-PGK were used to perform binding assays to plasminogen.

2.7 Interaction of anti-rGBS-PGK antibody with rGBS-PGK, PGK-M8 and PGK-M9 molecules.

Binding of the anti-rGBS-PGK antibody to PGK-M9, PGK-M8 and rGBS-PGK was measured by an ELISA binding assay as described previously (10). Decreasing concentrations of rGBS-PGK, PGK-M8, and PGK-M9 were diluted in 0.1 M sodium carbonate solution (pH 9.5) and passively adsorbed to wells of 96 well polystyrene plates incubated for 16 hours at 25^0C. Polystyrene plates were washed one time with 1 x tris buffered saline (TBS) for 10 minutes (pH 7.6) and blocked with the blocking buffer of 5% bovine serum albumin (BSA) (Sigma Aldrich) and 0.1% Tween-20 in 1xTBS for one hour at 25°C. The plates were washed for 10 minutes 3x with TBS and incubated with the primary antibody anti-rGBS-PGK (10) 50µl/well diluted 1:300 in the blocking buffer for one hour at 25°C. The plates were then washed 3x with TBS and incubated with the secondary antibody goat anti-rabbit-IgG alkaline phosphatase conjugate 50µl/well diluted 1:200 in the blocking buffer (10) for one hour at 25^0C. Plates were then washed 3 x with TBS and developed with 100µl of 4-nitrophenol phosphate (Sigma Aldrich) for 30 minutes in a dark room at 25^0C. After 30 minutes, the chemiluminescent reaction was stopped by adding 25µl NaOH (3N). Absorbance at 405 nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories; Mississauga, ON, CA).

2.8 Binding of PGK-M9, PGK-M8 and rGBS- PGK to immobilized plasminogen

Binding of PGK-M9, PGK-M8 , and rGBS- PGK to human plasminogen was measured by ELISA binding assays as previously described by Boone et al. (4) (Figure 2.7). Human plasminogen (0.1µg/well) (Sigma-Aldrich) diluted in 0.1 M sodium carbonate solution (pH 9.5) was passively adsorbed to wells of 96 well polystyrene plates by incubation for 8 hours at 25°C. Plates were washed one time with 1 x tris buffered saline (TBS) for 10 mintues (pH 7.6) and blocked with the blocking buffer (5% bovine serum albumin (BSA) (Sigma Aldrich) and 0.1% Tween-20 in 1xTBS) for 16 hours at 25°C. A plate containing no plasminogen was also blocked overnight in the same blocking buffer. After blocking, the plates were washed one time with 1 x TBS (100µl) and incubated with either 15µg/mL of PGK-M9, PGK-M8 or rGBS-PGK diluted in the blocking buffer for one hour at 25°C. The plates were washed for 10 minutes 3 x with TBS and incubated with the primary antibody anti-rGBS-PGK (10) 50µl/well diluted 1:300 in the blocking buffer for one hour at 25°C. Plates were then washed 3x with TBS and incubated with the secondary antibody goat anti-rabbit-IgG alkaline phosphatase conjugate (Sigma Aldrich) (50µl/well) diluted 1:200 in the blocking buffer (4) for one hour at 25°C. Plates were washed 3x with TBS and developed with 100µl of 4-nitrophenol phosphate (Sigma Aldrich) for 30 minutes in a dark room at 25°C. After 30 minutes, the chemiluminescent reaction was stopped by adding 25µl NaOH (3N). Absorbance at 405nm (A_{405}) was analyzed using an Athos LP400 microplate reader (Bio-Rad Laboratories; Mississauga, ON, CA).

Experiments were performed three different times in triplicate (a total of 9 times), and the A405 measurements from the plate without plasminogen were subtracted from the A_{405} measurements from the experimental plates with both plasminogen and rGBS-PGK, PGK-M8 and PGK-M9 to provide a final A_{405} measurement. These A_{405} absorbances were extrapolated from the standard curves containing decreasing amounts of rGBS-PGK, PGK-M8 and PGK-M9 to determine the amount of rGBS-PGK molecules remaining in the wells.

2.9 Assay to quantify PGK expressed on the surface of clinical isolates of GBS

The amount of PGK expressed on the surface of invasive GBS isolates was measured by using an ELISA assay previously described by Boone et al. (91). A random sample of GBS isolates from blood, body tissues and body fluids were assayed. These isolates had been submitted to the Provincial Laboratory in Edmonton for capsular serotyping between 2010 and 2011. GBS NCS13 is a serotype V GBS strain isolated from a leg wound. It has been shown to be a highly invasive isolate against HeLa cells (33). GBS NCS13 was used as a positive control for PGK expression; it has been shown to express PGK (91).

An *E.coli* strain obtained from frozen laboratory stocks was used as the negative control of surface GBS-PGK expression. A single colony of each isolate together with the positive and negative controls was inoculated into one ml Todd Hewitt broth and incubated overnight at 37°C. The overnight colonies were pelleted by centrifugation at 20 800 ×g for one minute. The supernatant was removed and one mL of 1x TBS was added to wash the bacterial pellet. The

pellet was washed with one ml of 1x TBS twice. After the washes, the supernatant was removed and the bacterial pellet was re-suspended in one ml TBS. In the next step, 100µl of the bacterial suspension was added to the wells of a 96-well polystyrene plate. The plate was covered with plastic wrap and incubated overnight (16 hours) at 4^0C to fix the bacteria to the wells. The next day, the wells were washed 1× with TBS (100 µl) and blocked with 5% BSA (200 µl) in TBS for 1 hour. After blocking, the wells were washed 3× with TBS (200 µl) and incubated 1 hour with anti-rGBS-PGK antibodies (50 µl; diluted1:300 in blocking buffer). The wells were washed 3× with TBS (200 µl) and incubated 1 hour with anti-rabbit IgG-alkaline phosphatase conjugate antibodies (50 µl; diluted 1:200 in blocking buffer; Sigma-Aldrich). The wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate (Sigma-Aldrich) for 30 minutes at room temperature before the reaction was stopped with 25 µl NaOH (3N). The absorbance at 405 nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories Ltd. Mississauga, ON, CA). The A_{405} values obtained were compared to the average A_{405} measurement from NCS13 cells grown for 20 h to determine the % A_{405}. All experiments were performed in triplicate.

2.10 **Statistical analysis**

Statistical analysis was performed for binding rGBS-PGK, PGK-M8 and PGK-M9 mutant molecules with plasminogen. The data was displayed in a column chart (Figure 3.8). The points represent the average value of all

experiments (performed in triplicate three times) and the error bars represent one standard deviation. The binding of PGK-M9 was compared to the binding of PGK-M8 and rGBS-PGK using the student's t-test, and a p value < 0.01 was considered statistically significant. The statistical analysis was done using Microsoft Excel 2007 software.

Table 2.1: *E.coli* strains used in this research investigation

E.coli strain	***Description***
JM101 pQE30	This strain of *E.coli* had the pQE30 vector plasmid. The JM101 possesses LacIq, a mutant lac repressor used to modulate lac expression. The pQE30 plasmid is maintained by carbenicillin. (Qiagen; Mississauga, ON).
M15 pREP4	Have the repressor plasmid pREP4, whose function is to express *lac* repressor, enabling *trans* repression of protein expression prior to IPTG induction. The pREP4 plasmid is maintained by kanamycin (Qiagen; Mississauga, ON).
DH5α pQE30-PGK-M8	The PQE30-PGK-M8 construct was cloned into DH5α strain. The plasmid is maintained by adding both carbenicillin and kanamycin. (10)

Table 2.2: PCR primers used in this research

PCR primer name	**Sequence**
PGK M7 (Forward)	5'-TTG AAG ATG TTG ACG GTG CGG CAG AAT CTG CGA ATG ACG CAG CAC TTG GTA AAT ACT GGG-3' (10)
PGK M7oL (Reverse)	5'-ACC GTC AAC ATC TTC AAA ACG AGT GTT TTC AAC C-3'(10)
PGK-Bam-F	5'-TTC **GGA TCC***GCT AAA TT GAC TGT TAA A-3'(64)
PGK-Pst-R	5'-TTC **CTG CAG*** TTA TTT TTC AGT CAA TGC-3' (64)

***** The *Bam* HI and *Pst* I sites are in bold.

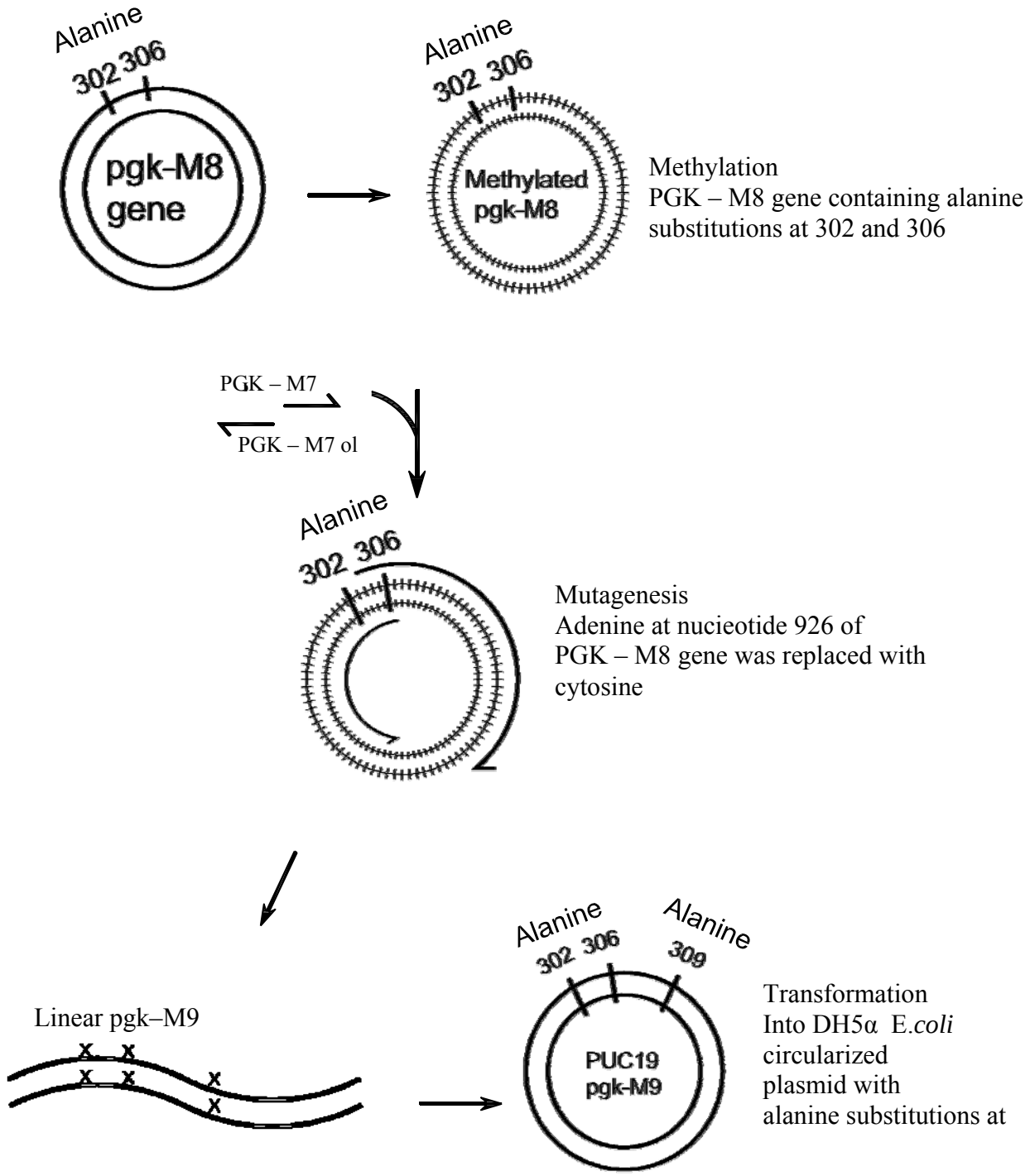


Figure 2.1: Site-directed mutagenesis of PGK-M8 to form PGK-M9

Table 2.3: Nucleotides that were changed by site-directed mutagenesis to form PGK-M9

Amino Acid Position	**PGK-wild type**		**PGK-M9**	
	Nucleotide	**Amino Acid**	**Nucleotide**	**Amino Acid**
302	AAA	Lysine	GCA	Alanine
306	AAA	Lysine	GCA	Alanine
309	GAA	Glutamic Acid	GCA	Alanine

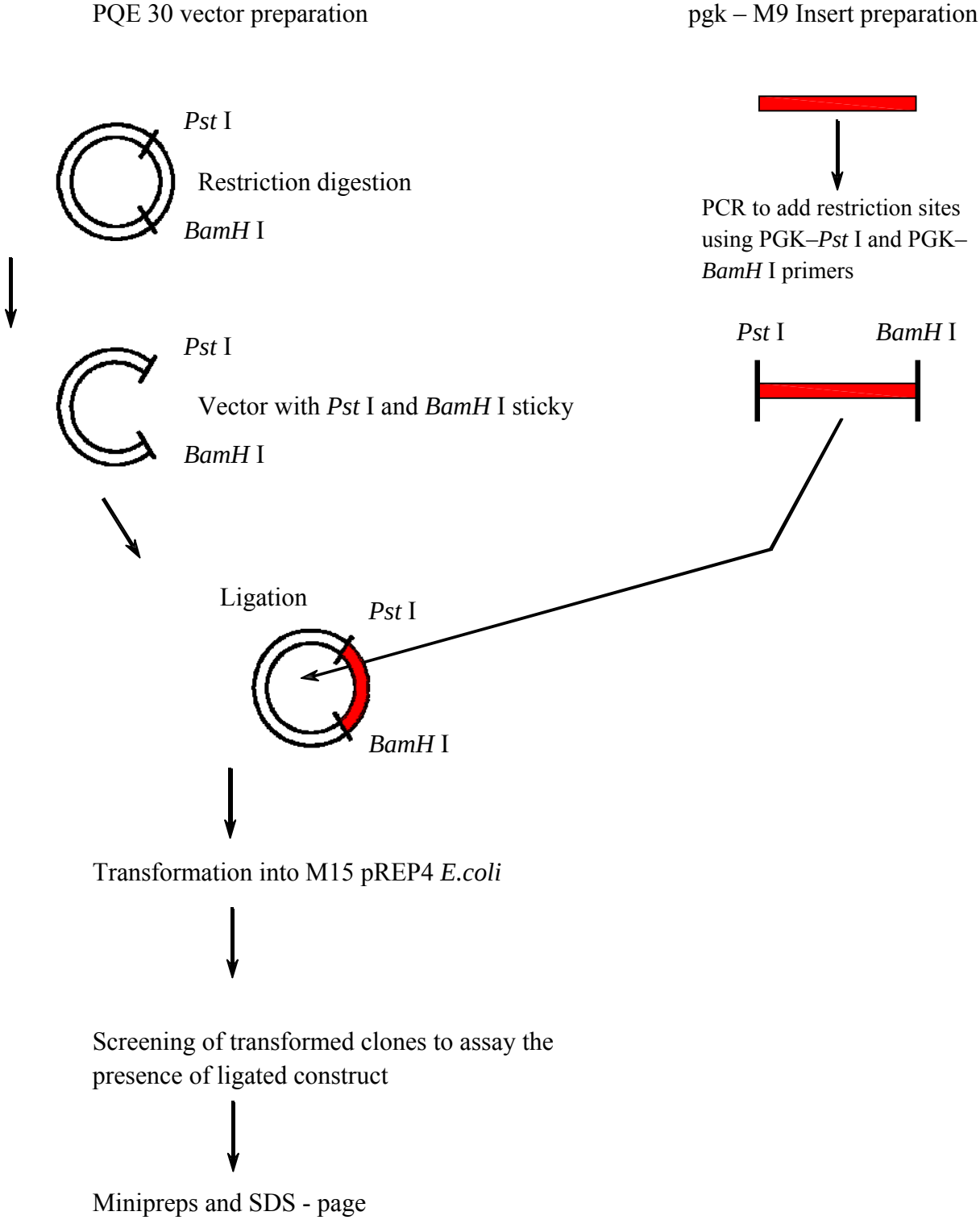


Figure 2.2: Cloning *pgk*-M9 sequence into pQE30 vector.

The *pgk*-M9 was cloned into the pQE30 expression vector (Figure 2.5) using the QIAexpressionist kit (Qiagen; Mississauga, ON, CA).

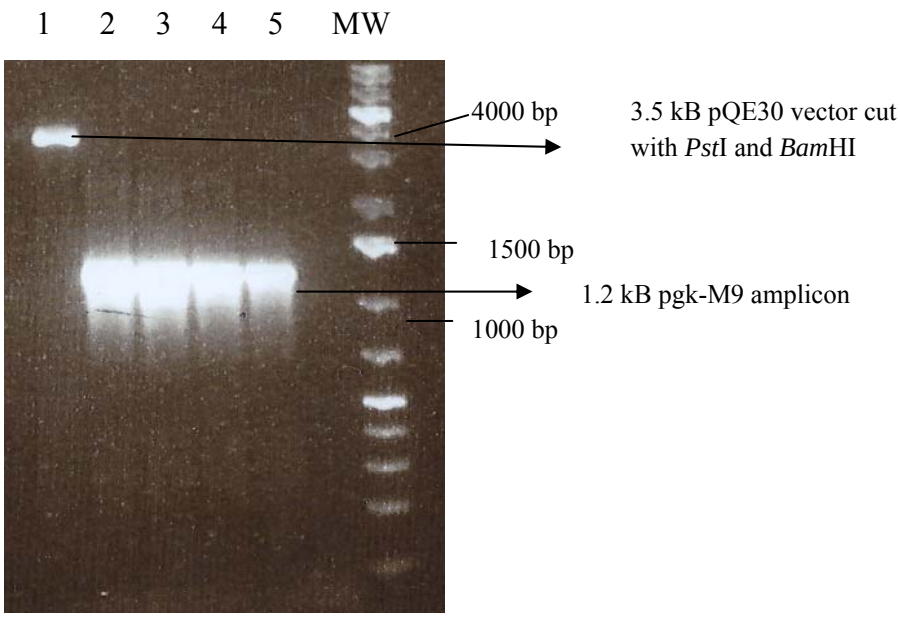


Figure 2.3: 1% agarose gel with vector pQE30 and *pgk*-M9 insert.

Five µl of the PCR reaction was electrophosed on a 1% agarose gel to check for purity of the reaction and if the amplicon was the correct size. The size of the pgk-M9 insert is 1200bp and the vector pQE30 is about 3461bp. Vector pQE30 in lane 1, and lanes 2, 3, 4 and 5 have the pgk-M9 insert.

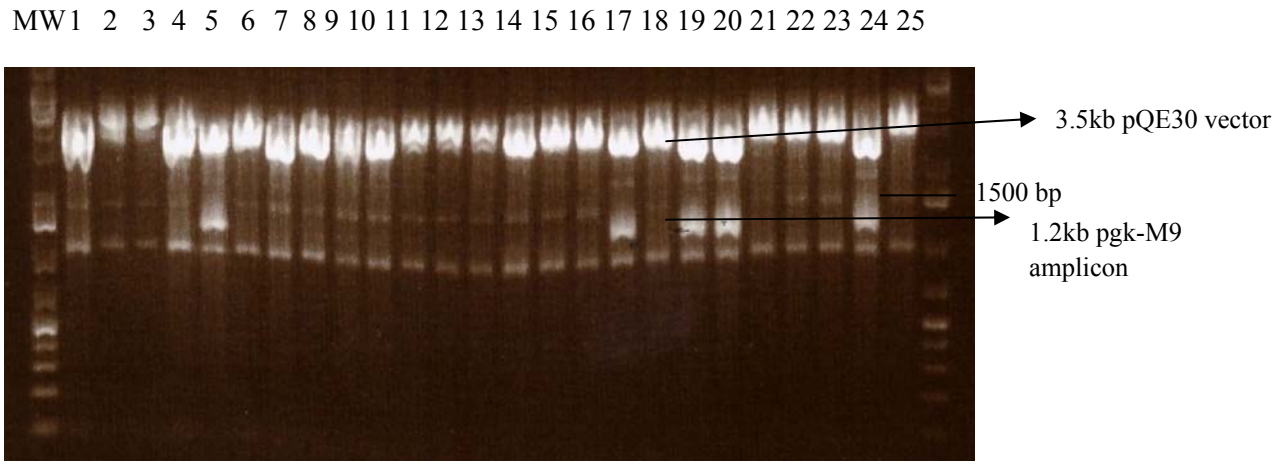


Figure 2.4 : Clones expressing recombinant protein PGK-M9

Transformed colonies were taken and added into the LB broth with kanamycin (25ug/ml) and carbenicillin (60ug/ml). The ligated construct was extracted and cut with *Pst*I and *Bam*HI and electrophosed on 1% agarose gel to analyze if the 1200 bp pgk-M9 amplicon and 3461bp pQE30 vector were present. The clones in lanes 5, 17, 19 and 20 contained both pQE30 (3461bp) and the pgk-M9 amplicon (1200 bp). These PGK-M9 clones were used to perform a small-scale rapid expression of protein using IPTG.

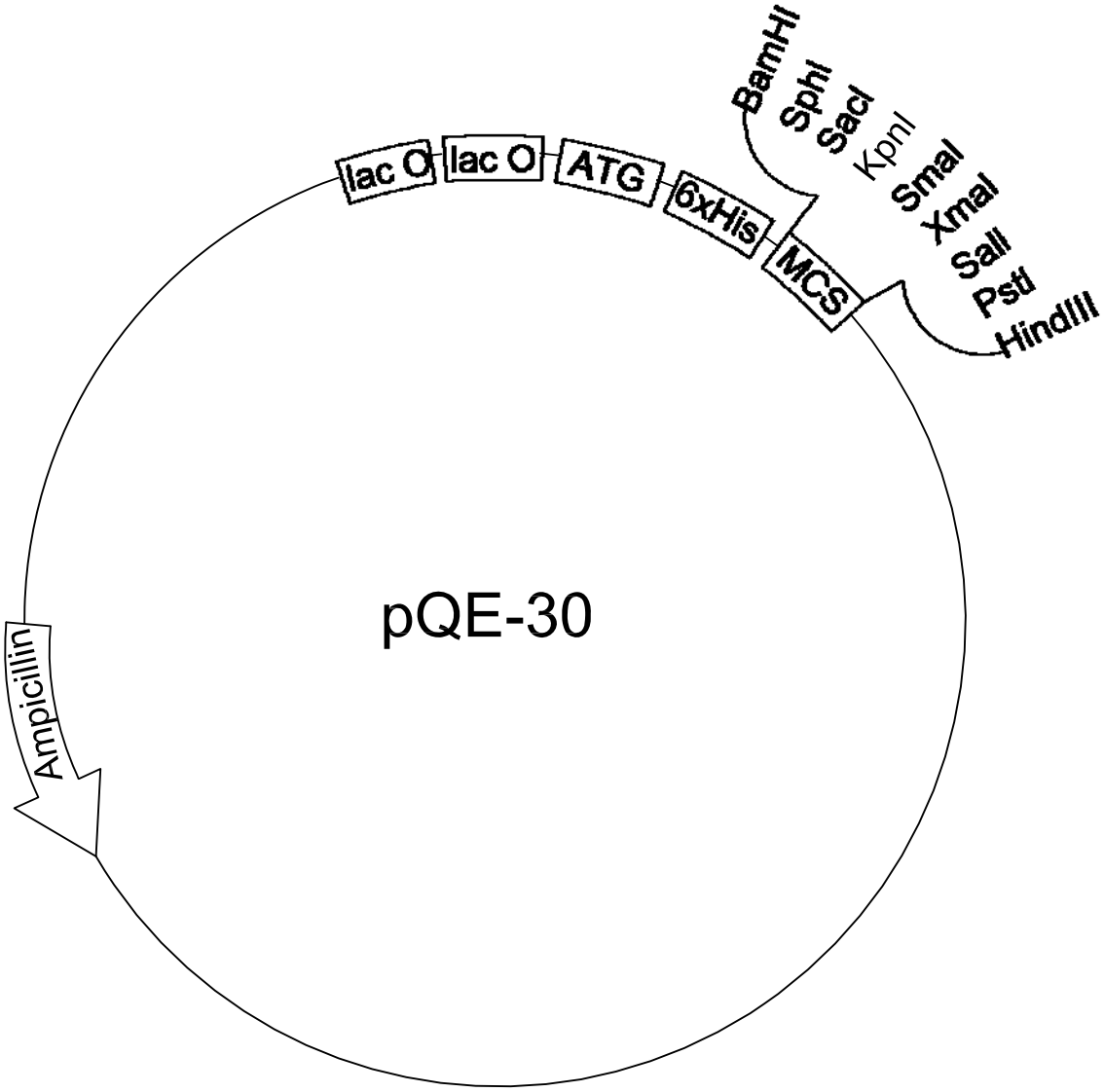


Figure 2.5: pQE30 vector features

lac O: lac operator sequences which increase lac repressor binding and ensure efficient IPTG protein expression. **ATG:** start codon, transcription takes place downstream of the start codon. **6xHis:** His tag sequence, allows protein purification using Ni-NTA method. **MCS:** multiple cloning sites, the *BamH*I and *PstI* sites were used for cloning of PGK-M9. **Ampicillin:** ampicillin resistance gene was used as an antibiotic selection marker during cloning.

PGK-M9 protein purification

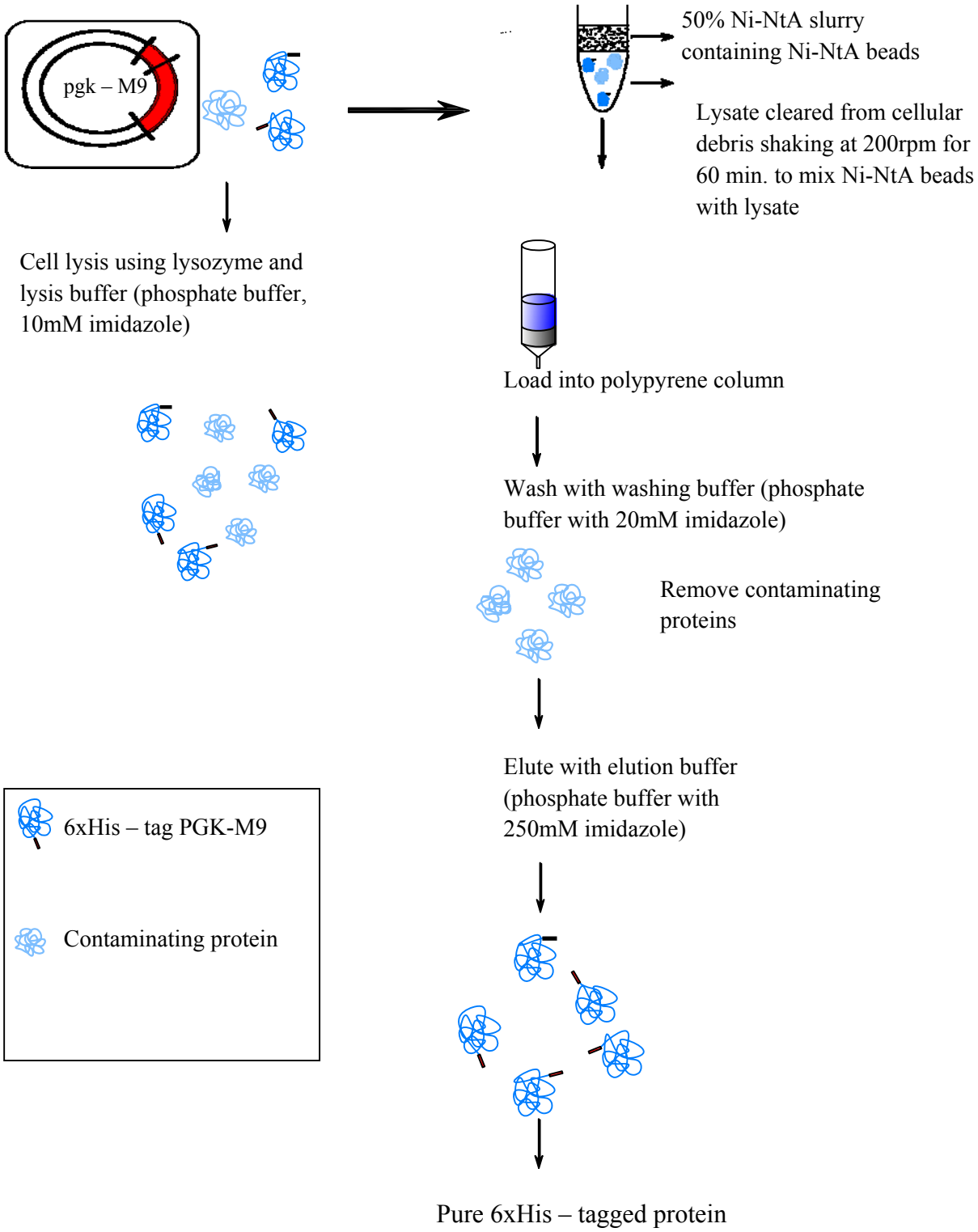


Figure 2.6: Protein purification

Protein purification of PGK-M9, PGK-M8 and rGBS-PGK was carried out under native conditions using the Qiaexpressionist kit (Qiagen; Mississauga, ON, CA) and methods described by Boone et al. (5)

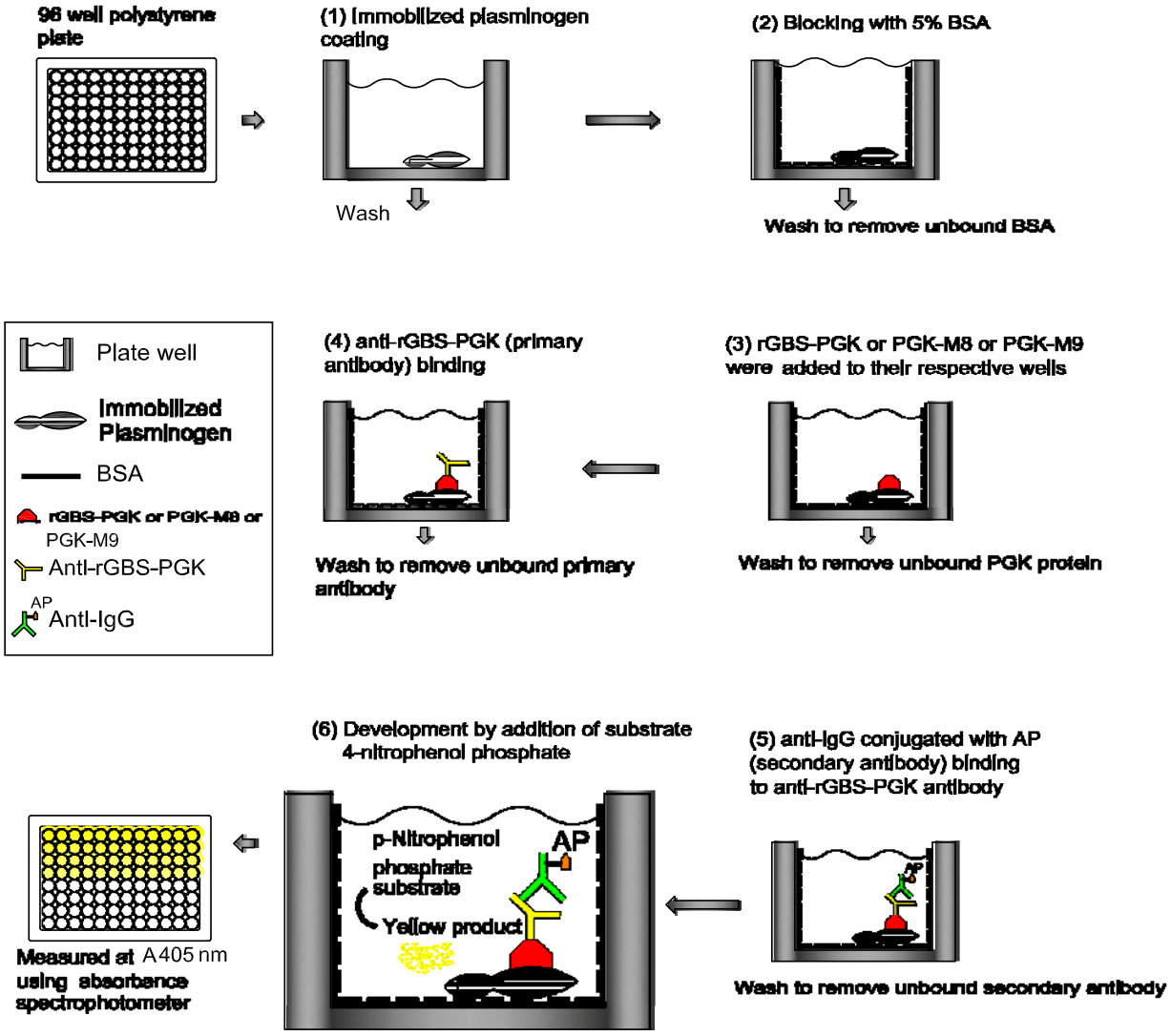


Figure 2.7: Binding of PGK-M9, PGK-M8 and rGBS- PGK to immobilized plasminogen

Binding of PGK-M9, PGK-M8, and rGBS- PGK to human plasminogen was measured by ELISA binding assays as previously described by Boone et al. (4)

Chapter 3:

Results

3.1 Site-directed mutagenesis

The *pgk* gene from the PGK-M8 mutant, which contains substitutions converting lysines 302 and 306 to alanines, was previously cloned into pUC19 (10). The mutant *pgk*-M8 gene was subjected to site-directed mutagenesis (Figure 2.1) following the procedure provided in the Gene Tailor Site-Directed Mutagenesis system (Invitrogen; Carlsbad, CA, USA). The mutagenesis assay resulted in an adenine-to-cytosine mutation at position 926 of the 1194-nucleotide-long *pgk*-M8 gene, which led codon GAA to change to GCA. This led to a mutation at amino acid 309 of PGK-M8, in which the glutamic acid was substituted for alanine. The GBS-PGK mutant construct, containing three alanine substitutions at positions 302, 306 and 309, was designated PGK-M9.

3.2 PGK-M9 cloning, protein purification and expression.

The cloning of the pgk-M9 amplicon into the pQE30 expression vector was done using the method described by Boone *et al*. for rGBS-PGK (5). The construct was transformed into M15 *E. coli,* resulting in the IPTG controlled expression of pgk-M9. To be certain the construct appeared as designed, clones were checked for the presence of both pQE30 and the pgk-M9 amplicon. Figure 2.4 shows the clones in lanes 5, 17, 19 and 20 contained both pQE30 (3461bp) and the pgk-M9 amplicon (1200 bp). These PGK-M9 clones were used to perform a small-scale rapid expression of protein using IPTG. The PGK-M9 clones 5 and 20 of figure 2.4 had high levels of expression and were subjected to protein purification. Figure 3.2 shows the crude IPTG-induced lysate (T4) and crude

non-induced lysate (T0) of PGK-M9 clones 5, 17, 19 and 20 in lanes one, two, three, and four respectively. The PGK-M9 band of size 42kDa is overexpressed in the IPTG-induced lysates. The clones 5 and 20 were subjected to protein expression as they visually appeared to express the greatest amount of PGK. PGK-M8 (Figure 3.4) and rGBS-PGK (Figure 3.3) from frozen laboratory stocks were also subjected to protein expression and purification. Figure 3.5 shows the purified eluates of PGK-M9 together with crude lysates. The lanes containing the purified PGK proteins show only one 42kDa band and no other contaminating proteins.

3.3 Glycolytic activity of PGK-M9, rGBS-PGK, and PGK-M8

The purified PGK-M9 and PGK-M8 glycolytic activity was compared to the glycolytic activity of rGBS-PGK. The glycolytic activity of rGBS-PGK was comparable to the activity of the positive control PGK derived from *Sacchromyces cerevisiae*. PGK-M9 and PGK-M8 demonstrated activities of 129% and 149% compared to the glycolytic activity of rGBS-PGK (Figure 3.6). The glycolytic enzyme activity of negative control potassium phosphate was 0%.

3.4 Binding of PGK-M9, rGBS-PGK, and PGK-M8 to plasminogen

As previously described, ELISA was used to assay the ability of PGK-M9, PGK-M8 and rGBS-PGK to bind to immobilized plasminogen (4). As shown in figure 3.8, substituting lysine 302, lysine 306 and glutamic acid 309 to alanine in PGK-M9 significantly ($p<0.01$) reduced PGK's ability to bind to plasminogen. The amount of rGBS-PGK, PGK-M8 and PGK-M9 that remained bound to

plasminogen after incubation in wells containing immobilized plasminogen was 4.40ng, 2.74ng and 0.23ng respectively. The binding of PGK-M9 to plasminogen is significantly ($p<0.01$) reduced compared to PGK-M8 and rGBS-PGK. This indicates that the position 309 substitution of glutamic acid residue to alanine plays an important role in binding GBS-PGK to plasminogen. The three substituted alanine at amino acid positions -- 302, 306 and 309 -- were highlighted (Figure 3.1).

3.5 Measuring the expression of PGK in GBS from various clinical isolates

As previously described, an ELISA assay was used to measure the amount of PGK expressed on the surface of various clinical isolates of GBS (10). The PGK expression in positive control NCS13 had an average absorbance of 0.89 (Figure 3.9). The negative control for PGK expression *E. coli* had an absorbance of 0.10 (Figure 3.9). The GBS isolates from various sources (including blood, body tissues and body fluids) had PGK expression levels close to that of the positive control GBS NCS13 (Figure 3.9). This analysis showed that all isolates of GBS causing invasive disease have surface GBS-PGK.

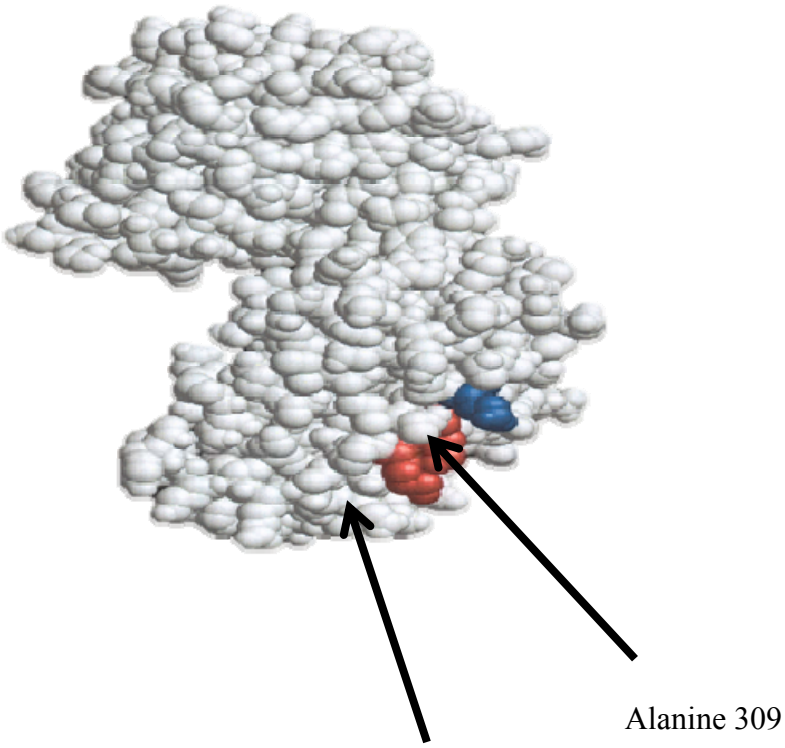


Figure 3.1: A space-filling model of PGK-M9

This PGK-M9 model highlights the location of the three alanine substitutions which were visualized using RasMol. The image shows alanine substitutions 302, 306 (red) (10) and alanine substitution 309 (blue) in PGK-M9.

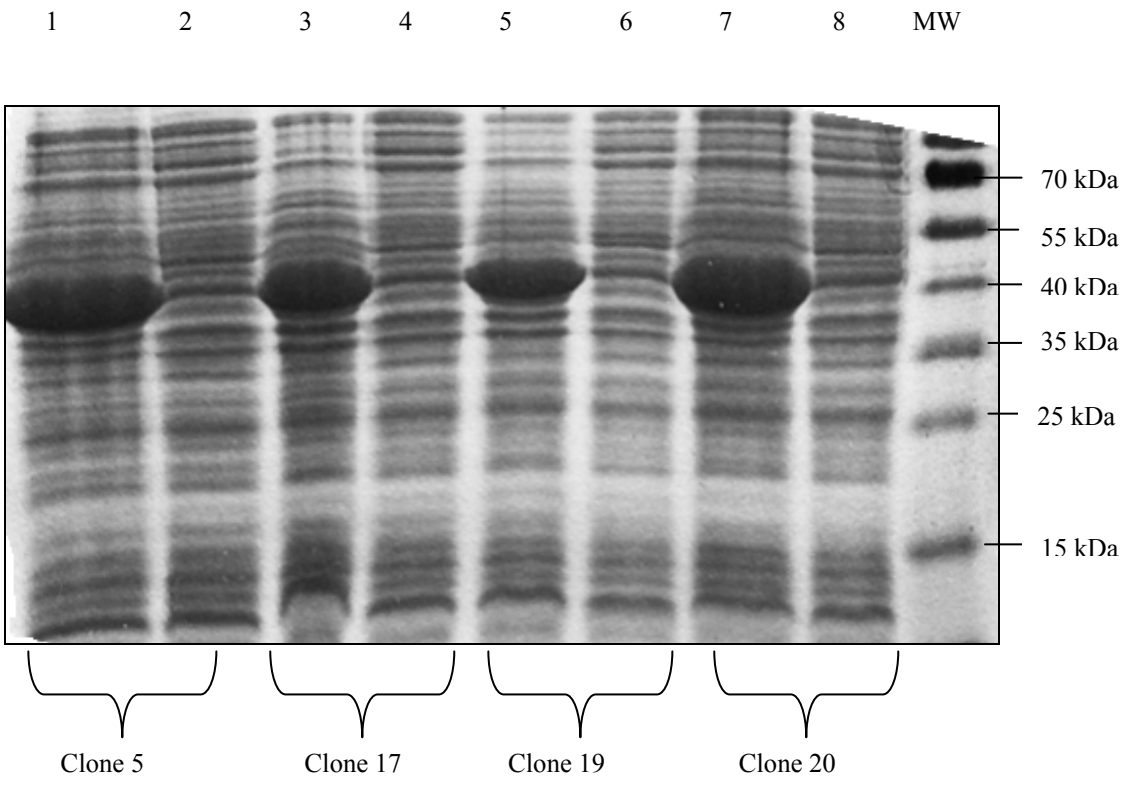


Figure 3.2: Small-scale rapid expression of PGK-M9 in *E.coli* M15

The clones in lanes 5, 17, 19 and 20 (Figure 2.4) were used to perform small-scale rapid expression of protein using IPTG induction. The PGK-M9 clones 5 and 20 of figure 2.4 had a high level of protein expression and were subjected to protein purification. This figure illustrates the crude IPTG induced lysate (T= 4 hours), and crude non-induced lysate (T0) of PGK-M9 clones 5, 17, 19 and 20 in lanes one, two, three, and four respectively. The PGK-M9 band of size 42kDa is overexpressed in the IPTG-induced lysates. The clones 5 and 20 were subjected to protein expression.

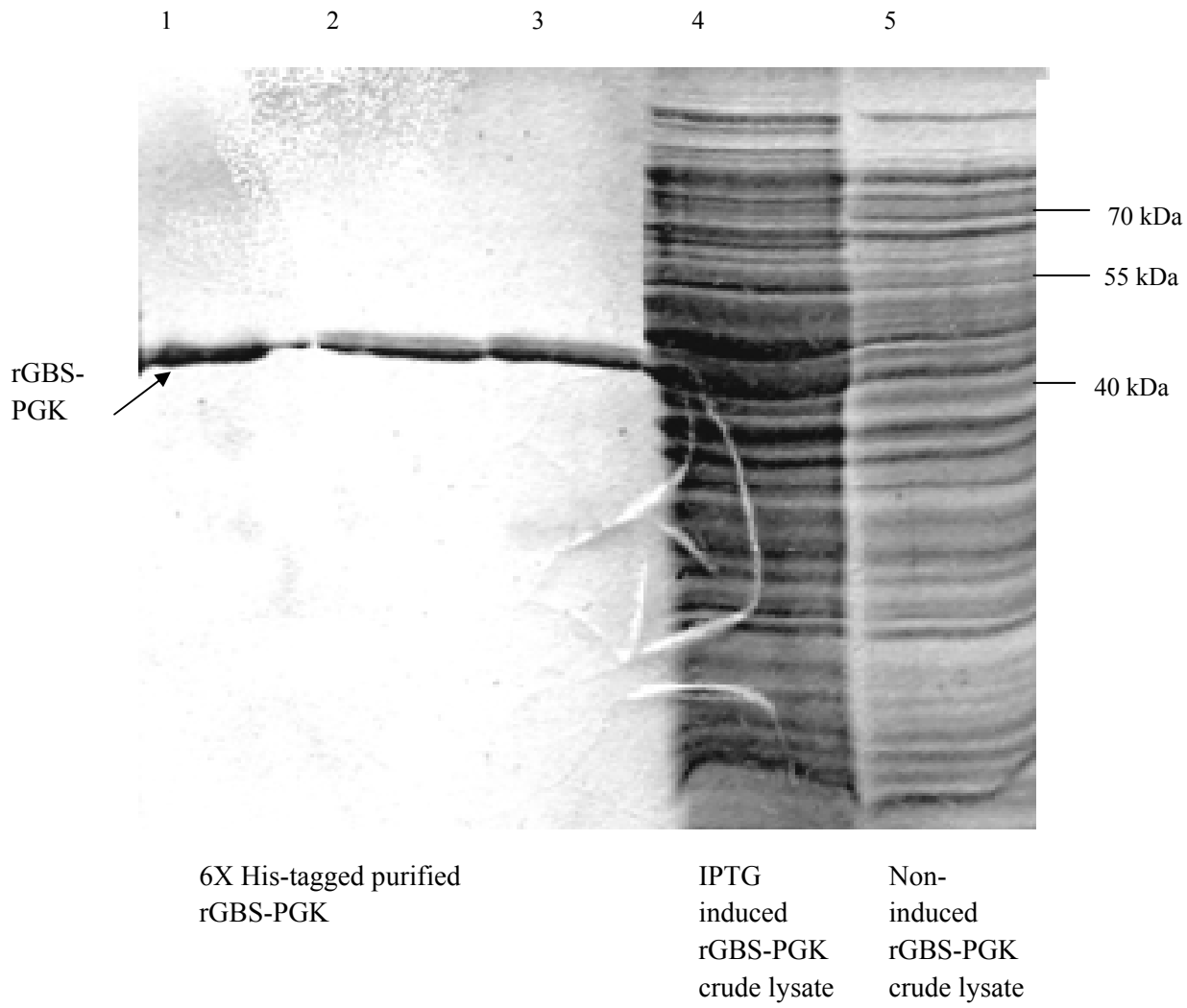


Figure 3.3. Expression and purification of rGBS-PGK in *E. coli* M15.

rGBS-PGK expression was induced in *E.coli* M15[pREP4][pQE30-rGBS-PGK] (5) using IPTG (2mM). In lanes 1, 2, and 3, the purified rGBS-PGK is illustrated. The IPTG induced and non-induced rGBS-PGK crude cell lysates in lanes 4 and 5 are shown. The 42kDa rGBS-PGK band is overexpressed in the IPTG-induced lysate.

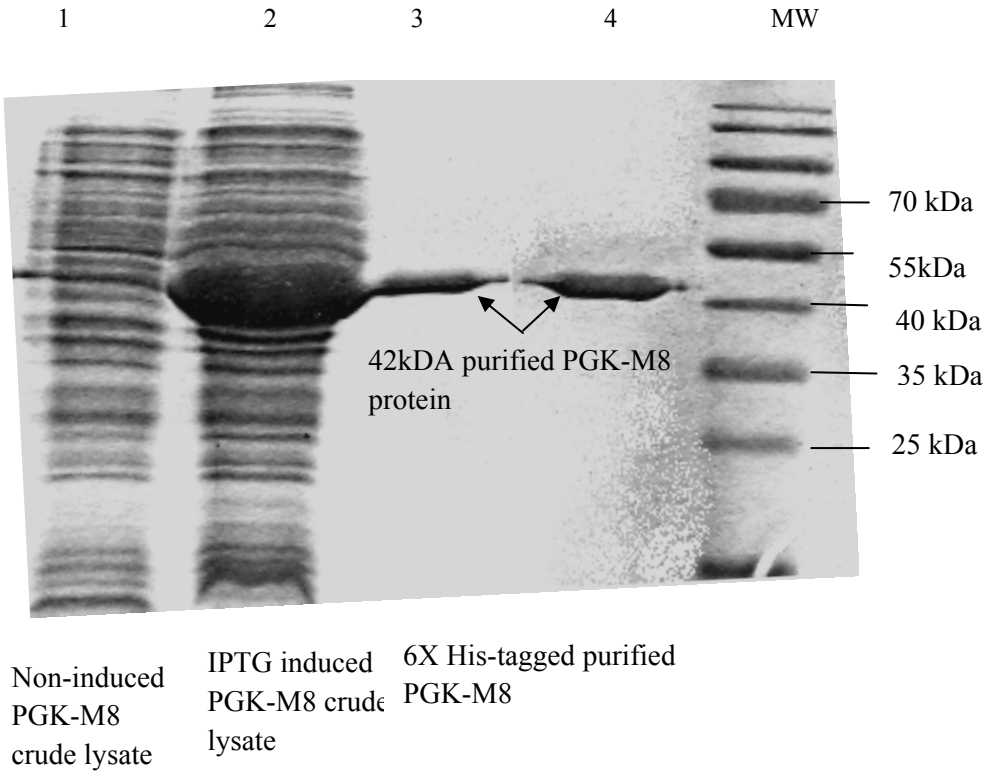


Figure 3.4. Expression and purification of PGK-M8 in *E.coli* M15.

PGK-M8 was expressed in *E.coli* M15[pREP4][pQE30-pgk-M8] (5,10) using IPTG (2mM) induction. In lanes 1 and 2, the IPTG non-induced PGK-M8 and induced PGK-M8 crude cell lysates are shown. In lanes 3 and 4, two eluates of purified PGK-M8 are illustrated. The 42kDa PGK-M8 band is overexpressed in the IPTG-induced lysate. The PageRuler prestained protein ladder was used to analyze the size of proteins.

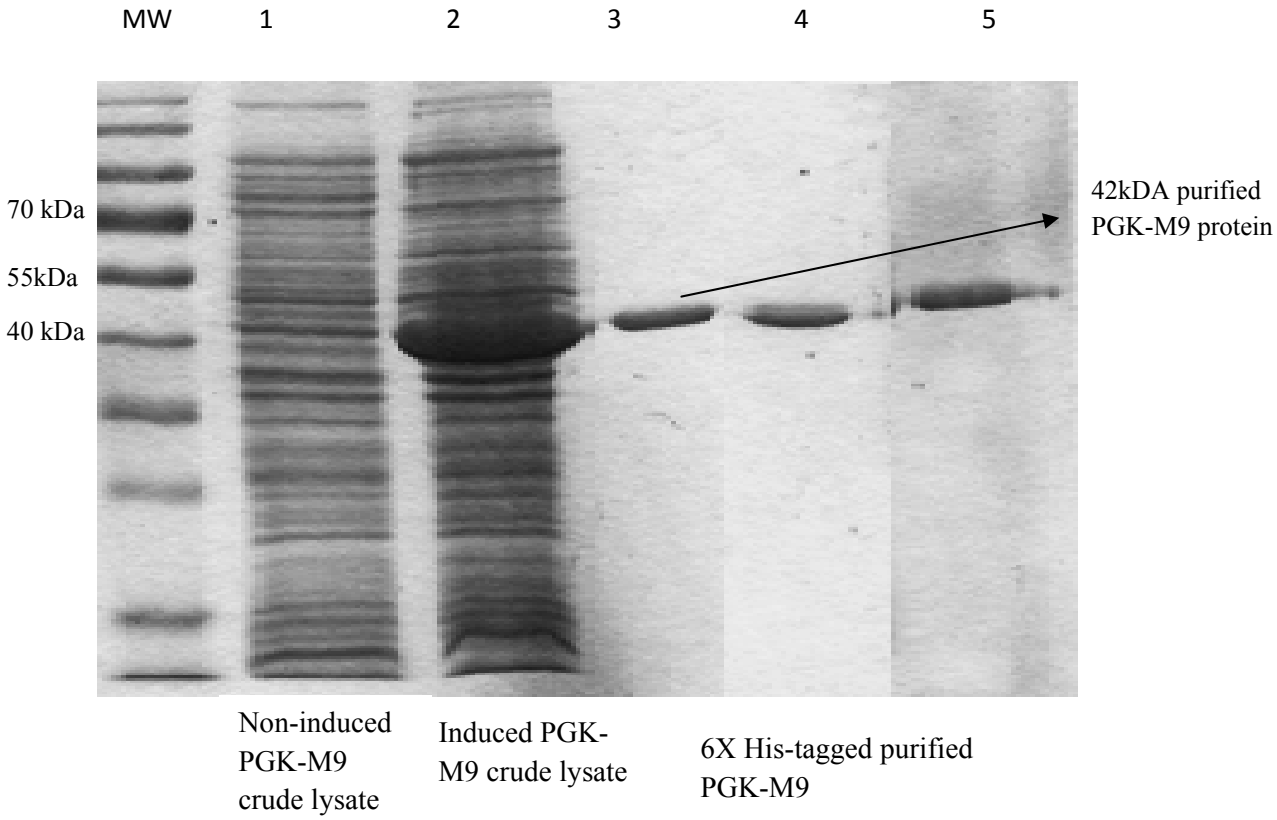


Figure 3.5. Expression and purification of PGK-M9 in *E.coli* M15.

PGK-M9 was expressed in *E.coli* M15[pREP4][pQE30-pgk-M9] using IPTG (2mM) induction. In lanes 1 and 2, the IPTG non-induced PGK-M9 and induced PGK-M8 crude cell lysates are shown. In lanes 3 ,4, and 5, eluates of purified PGK-M9 are illustrated. The 42kDa PGK-M9 band is overexpressed in the IPTG-induced lysate. The PageRuler pre-stained protein ladder was used to analyze the size of proteins.

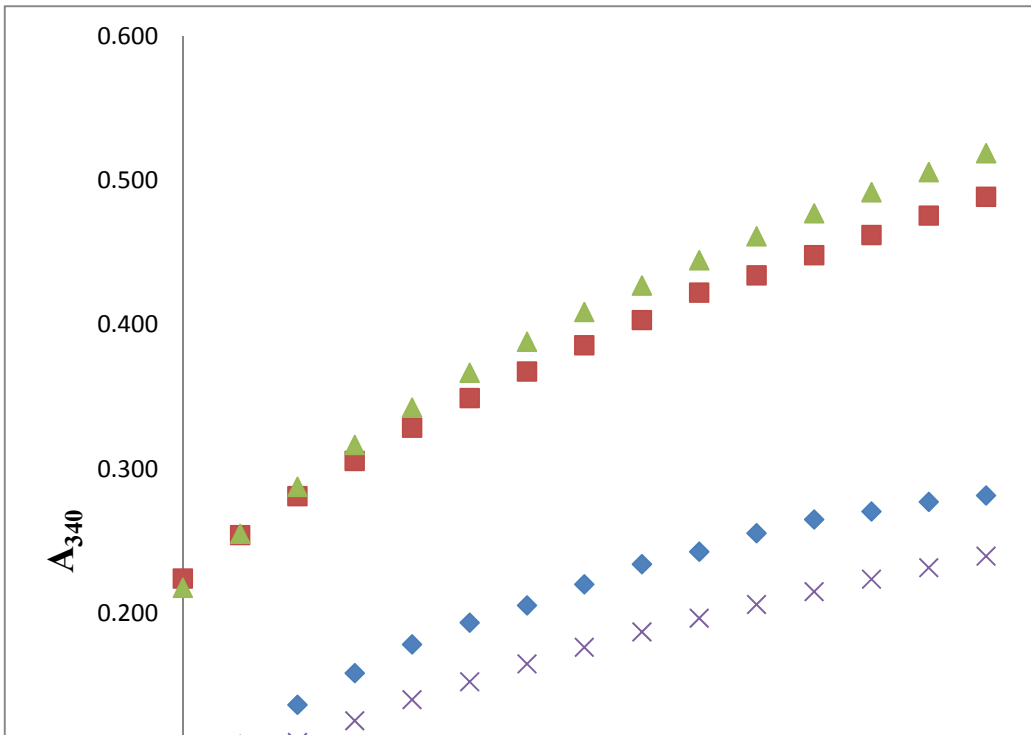


Figure 3.6: Enzymatic activities of rGBS-PGK, PGK-M8 and PGK-M9

Mutant PGK-M9, PGK-M8 and rGBS-PGK molecules were expressed in M15 and purified using the 6x His-tag Qiagen Qiaxpressionist system. The glycolytic activity of PGK-M9 was measured as previously described (106), (10). The glycolytic activity of PGK-M9 was compared to the glycolytic activity of positive control PGK (0.06 units) (Sigma Aldrich, St. Louis, MO, USA) purified from *Saccharomyces cerevisiae* (PGK-sc) and to the glycolytic activity of PGK-M8 and to that of rGBS-PGK. The data points above are representative of experiments performed in duplicate. The PGK-M9 and PGK-M8 demonstrated activities of 129% and 149% compared to the glycolytic activity of rGBS-PGK. The glycolytic enzyme activity of negative control potassium phosphate was 0%.

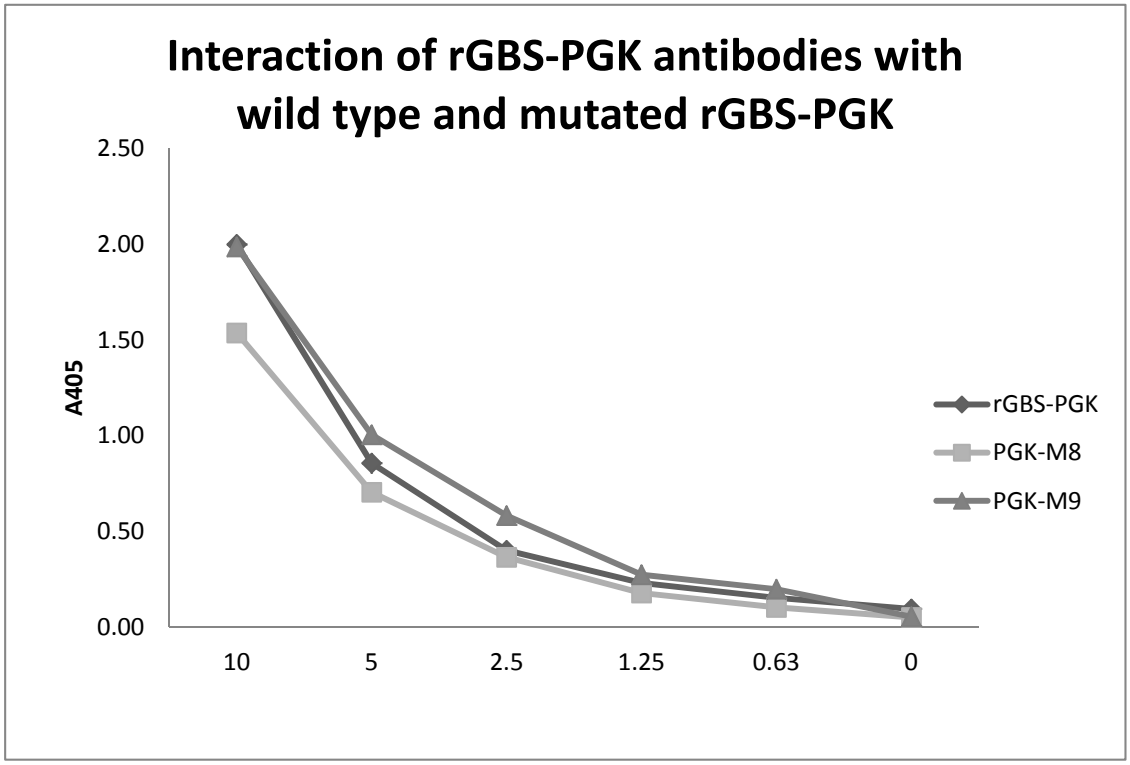
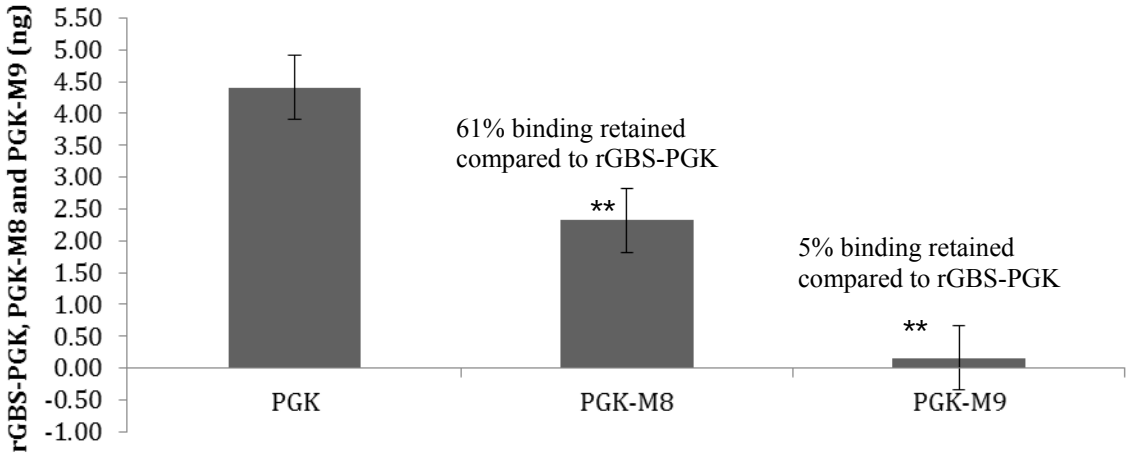


Figure 3.7: Standard curves for PGK ELISA assays.

As previously described, ELISA binding assays were used to measure the binding of the anti-rGBS-PGK antibody to PGK-M9, PGK-M8 (10) and rGBS- PGK (10). Data points on the above graph are the average A_{405} values from assays performed in triplicate. The A_{405} values of the plasminogen binding assay to determine the amount of rGBS-PGK, PGK-M8 and PGK-M9 molecules remaining bound to plasminogen were extrapolated from the above standard curves.

Figure 3.8: **Binding of PGK-M9, PGK-M8 and rGBS-PGK to immobilized plasminogen**



ELISA binding assays were used to measure the binding of PGK-M9, PGK-M8, and rGBS- PGK to human plasminogen (4). Substituting lysine 302, lysine 306 and glutamic acid 309 for alanine in PGK-M9 significantly ($p<0.01$) reduced the PGK's binding ability to plasminogen. The amount of rGBS-PGK, PGK-M8 and PGK-M9 that remained bound to plasminogen after incubation in wells containing immobilized plasminogen was 4.40ng, 2.74ng and 0.23ng respectively. The binding of PGK-M9 to plasminogen is significantly ($p<0.01$) reduced compared to the plasminogen binding ability of PGK-M8 and rGBS-PGK. Data points represent the average values from assays performed three times in triplicate; error bars represent one standard deviation ** , p-value <0.01.

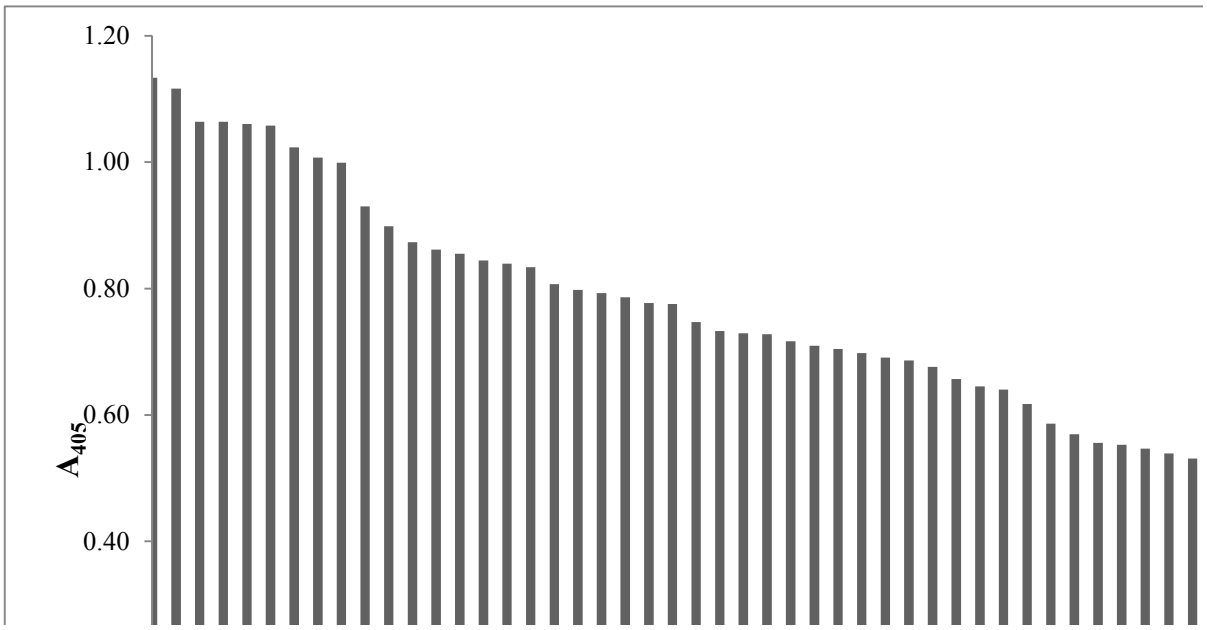


Figure 3.9: Measuring the expression of PGK in invasive GBS isolates

As described by Boone et al. (91), an ELISA assay was used to measure the amount of PGK expressed on the surface of invasive GBS isolates. GBS isolates were collected from blood, body tissues and body fluids submitted to the Provincial Laboratory for Public Health Edmonton for serotyping between 2010 and 2011. GBS NCS13 was used as a positive control for PGK expression (91). The *E. coli* strain obtained from frozen laboratory stocks was used as a negative control of surface GBS-PGK expression. All data points above represent experiments performed in triplicate. The PGK expression in positive control NCS13 had an average absorbance of 0.89. The negative control for PGK expression of *E. coli* had an absorbance of 0.10. The GBS isolates from various sources (including blood, body tissues and body fluids) had PGK expression levels close to those of the positive control GBS NCS13. This analysis showed that all isolates of GBS causing invasive disease have surface GBS-PGK.

Chapter 4:

Discussion and Conclusion

The glycolytic enzyme PGK on the surface of GBS is unusual. Surface expression of this enzyme suggests it may have more than a glycolysis role. Previous studies have shown that GBS-PGK binds to actin and plasminogen (5). As shown previously, surface-expressed PGK was present on the surface of all GBS isolates examined in an ELISA assay (Figure 3.9). This would suggest that where this enzyme is placed on the cell surface plays an important role in invasive GBS. My results and those of previous studies strongly suggest this role involves binding to plasminogen.

To date, three surface proteins identified on the surface of GBS have been shown to bind to? plasminogen: GAPDH (101), Skizzle (100) and PGK (5). Plasminogen binding to the bacterial surface is a property demonstrated by many bacteria (4-8) and fungi (9). This property is believed to be a key factor that helps the bacteria to invade further into the host by destroying host barriers. Human plasminogen is a 92 kDa protein that circulates in human plasma. Plasminogen in humans is normally activated by two plasminogen activators: urokinase plasminogen activator (upA) and tissue-type plasminogen activator (tpA)(8). The PGK-plasminogen complex may be able activate plasmin using tpA and upA. An example of this strategy is the GBS Skizzle protein (100). The Skizzle protein was shown to bind to plasminogen and then activate to plasmin by human upA and tpA (100) . Another example, GAPDH, binds to plasminogen. The GAPDH-plasminogen complex activates plasmin using tpA and upA, which promotes GBS invasion as shown in a mouse model (101). It has been previously shown that GBS-PGK binds fibrinogen and fibrin *in vitro*. Fibrinogen is broken down into

fibrin by plasmin. GBS-PGK may form a complex with fibrinogen and may further recruit plasminogen. The plasminogen may be activated to plasmin by tpA and upA, causing the fibrinogen to break down to fibrin.

Previous investigations in our laboratory created mutant GBS-PGK molecules that displayed reduced actin and plasminogen binding (4). These investigations found that the major plasminogen-binding domains were located between amino acids 126-130, 154-165, 202-205, 207-221, and 291-305 of the GBS PGK molecule, which is 398 amino acids long (4). To further augment these studies, the negatively charged glutamic acid residue, 309, was targeted for an alanine substitution. Glutamic acid 309 has been shown to localize near the 302 and 306 alanine residues (substituted from lysine) of PGK-M8, a mutant displaying a reduction in plasminogen binding (4). It has also been shown that 6-ACA (lysine analogue) blocked the rGBS-PGK binding to plasminogen, indicating that lysine residues are needed to bind rGBS-PGK to plasminogen (5). However, the mutant PGK-M8 with only lysine-to-alanine substitutions had greater plasminogen binding compared with triple mutant PGK-M9. The creation of PGK-M9, a mutant GBS-PGK molecule with three alanine substitutions at sites 302, 306, and 309, was constructed with the objective of negating plasminogen binding yet retaining glycolytic enzyme function. The glutamic acid residue 309 can be seen to localize near lysine residues 302 and 306 in the PGK-M9 space-filling model (Figure 3.1). Peptide mapping has shown that lysine residues 302 and 306 localized near the glutamic acid residues 286 and 288 (4). In PGK-M9, converting the lysine and glutamic acid residues to alanines diminished the

plasminogen binding. This suggests that negatively charged glutamic acid residues, together with lysine residues, may be involved in plasminogen binding. A mutant molecule that contains only glutamic acid 309 to alanine substitution may have higher or lower binding to plasminogen. However, it is most likely that this mutant may have higher plasminogen binding than PGK-M9, since the lysine residues at 302, 306 of the GBS-PGK are still functional and may be able to bind plasminogen.

It has been previously shown that negatively charged residues like aspartic acid were required by the α-enolase molecule from S. *pneumoniae*. In this study it was demonstrated that substituting negatively charged aspartic acid with alanine abolished α-enolase binding to plasminogen (103). The results presented in this thesis demonstrate a similar finding. Substituting a negatively charged amino acid (glutamic acid 309) with alanine diminished the binding of PGK-M9 to human plasminogen. PGK-M9 has reduced binding to plasminogen compared with PGK-M8 and wild-type rGBS-PGK. The mutant PGK-M9 had similar binding to anti-rGBS-PGK polyclonal antibody as PGK-M8 and rGBS-PGK (Figure 3.7), and from this we may be able to conclude that any change in binding of the mutant to plasminogen is due to the amino acid substitution and not to the polyclonal antibodies' failure to recognize PGK-M9. The results presented in this thesis further indicate that this mutant PGK-M9 was glycolytically active. The PGK-M9 and PGK-M8 demonstrated activities of 129% and 149% compared to the glycolytic activity of rGBS-PGK (Figure 3.6). The glycolytic enzyme activity of the negative control, potassium phosphate, was 0%. The glycolytic

enzyme activity of both PGK-M9 and PGK-M8 is higher than 100%. A possible explanation for this could be that the alanine substitutions increased the PGK enzymatic activity. Since PGK-M9 is glycolytically active, this may also mean that the three alanine substitutions, 302, 306 and 309, are not present in the enzymatic cleft of the PGK-M9 molecule. Previously, PGK-M2, a mutant GBS-PGK molecule created by lysine 204 and 208 substitutions to alanine, had only 27% enzyme activity (4). It is important that PGK-M9 is glycolytically active as GBS-PGK is an enzyme essential for glycolysis (Figure 1.5). Additionally, since there is only one chromosomal copy of the *pgk* gene (97,102), a non-functional GBS-PGK means a GBS strain made using this *pgk gene* will not be able to survive due to lack of glycolysis.

The strong reduction in plasminogen binding by PGK-M9 raises the possibility of using this protein as a possible vaccine component. GBS-PGK is a good candidate for multiple reasons. Previously, mice inoculated with whole GBS-PGK antibodies were shown to be protected against lethal GBS infection (3). In another study, S. p*neumoniae* GAPDH antibodies were shown to protect against S. *pneumoniae* infection in mice. The purified PGK-M9 molecule can be used to immunize mice. Mouse antibodies can be elicited to PGK-M9 and can be tested for cross reactivity with different serotypes of GBS *in vitro*. PGK-M9 antibodies can be injected into neonatal mice, and when the mice are challenged with GBS infection, the PGK-M9 should provide protection. To further demonstrate PGK's role *in vivo,* the GBS *pgk* gene can be replaced with pgk-M9 gene. The virulence of this strain lacking plasminogen binding can be measured

using animal models as previously described (107). PGK on the surface of GBS is thought to act as a plasminogen receptor, and has been shown to bind plasminogen *in vitro* (5). Plasminogen binding *in vivo* has not yet been shown. However, if a GBS strain lacking plasminogen-binding PGK is created, the plasminogen will not be activated to plasmin. If plasminogen is not activated to plasmin, the extracellular matrix may not degrade.

In conclusion, the PGK-M9 molecule was created using the previously described GBS-PGK mutant PGK-M8 (containing lysine to alanine at 302 and 306) (10). Additional substitutions in PGK-M8, converting the glutamic acid residue 309 to alanine as hypothesized, reduced the PGK binding of plasminogen to negligible. The PGK-M9 molecule can be used to enhance our understanding of GBS-PGK's role with respect to plasminogen binding in GBS pathogenesis.

4.1 References:

(1) Doran KS, Nizet V. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol 2004 Oct;54(1):23-31.

(2) Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep 2010 Nov 19;59(RR-10):1-36.

(3) Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, Younes ZN, et al. Identification of major outer surface proteins of Streptococcus agalactiae. Infect Immun 2002 Mar;70(3):1254-1259.

(4) Boone TJ, Tyrrell GJ. Identification of the actin and plasminogen binding regions of group B streptococcal phosphoglycerate kinase. J Biol Chem 2012 Aug 17;287(34):29035-29044.

(5) Boone TJ, Burnham CA, Tyrrell GJ. Binding of group B streptococcal phosphoglycerate kinase to plasminogen and actin. Microb Pathog 2011 Jun 28.

(6) Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S. alpha-Enolase of Streptococcus pneumoniae is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. Mol Microbiol 2001 Jun;40(6):1273-1287.

(7) Bergmann S, Rohde M, Hammerschmidt S. Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pneumoniae is a surface-displayed plasminogen-binding protein. Infect Immun 2004 Apr;72(4):2416-2419.

(8) Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol 2012;2012:482096.

(9) Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA. Candida albicans binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol 2003 Mar;47(6):1637-1651.

(10) T. J. Boone. Characterization of Phosphoglycerate Kinase Expressed on the Surface of Group B StreptococcusUniversity of Alberta; 2012.

(11) Mahon CR, Lehman CD, Manuselis G. Textbook of Diagnostic Microbiology. Philadelphia, USA: Saunders Elsevier; 2007.

(12) Lancefield RC. A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci. J Exp Med 1933 Mar 31;57(4):571-595.

(13) Tyrrell GJ, Senzilet LD, Spika JS, Kertesz DA, Alagaratnam M, Lovgren M, et al. Invasive disease due to group B streptococcal infection in adults: results from a Canadian, population-based, active laboratory surveillance study--1996. Sentinel Health Unit Surveillance System Site Coordinators. J Infect Dis 2000 Jul;182(1):168-173.

(14) Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, et al. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis 1998 Apr;177(4):998-1002.

(15) Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis 2005 Sep 15;41(6):839-847.

(16) Bauserman MS, Laughon MM, Hornik CP, Smith PB, Benjamin DK,Jr, Clark RH, et al. Group B Streptococcus and Escherichia coli Infections in the Intensive Care Nursery in the Era of Intrapartum Antibiotic Prophylaxis. Pediatr Infect Dis J 2013 Mar;32(3):208-212.

(17) Goluszko P, Popov V, Wen J, Jones A, Yallampalli C. Group B streptococcus exploits lipid rafts and phosphoinositide 3-kinase/Akt signaling pathway to invade human endometrial cells. Am J Obstet Gynecol 2008 Nov;199(5):548.e1-548.e9.

(18) Deutscher M, Lewis M, Zell ER, Taylor TH,Jr, Van Beneden C, Schrag S, et al. Incidence and severity of invasive Streptococcus pneumoniae, group A Streptococcus, and group B Streptococcus infections among pregnant and postpartum women. Clin Infect Dis 2011 Jul 15;53(2):114-123.

(19) Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, et al. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis 2009 Jul 1;49(1):85-92.

(20) Farley MM. Group B streptococcal disease in nonpregnant adults. Clin Infect Dis 2001 Aug 15;33(4):556-561.

(21) Anthony BF, Okada DM, Hobel CJ. Epidemiology of group B Streptococcus: longitudinal observations during pregnancy. J Infect Dis 1978 May;137(5):524-530.

(22) Ulett GC, Webb RI, Ulett KB, Cui X, Benjamin WH, Crowley M, et al. Group B Streptococcus (GBS) urinary tract infection involves binding of GBS to

bladder uroepithelium and potent but GBS-specific induction of interleukin 1alpha. J Infect Dis 2010 Mar 15;201(6):866-870.

(23) Kahlke V, Jongen J, Peleikis HG, Herbst RA. Perianal streptococcal dermatitis in adults: its association with pruritic anorectal diseases is mainly caused by group B streptococci. Colorectal Dis 2012 Dec 5.

(24) Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, et al. Genome analysis reveals pili in Group B Streptococcus. Science 2005 Jul 1;309(5731):105.

(25) Rosini R, Rinaudo CD, Soriani M, Lauer P, Mora M, Maione D, et al. Identification of novel genomic islands coding for antigenic pilus-like structures in Streptococcus agalactiae. Mol Microbiol 2006 Jul;61(1):126-141.

(26) Maisey HC, Doran KS, Nizet V. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert Rev Mol Med 2008 Sep 22;10:e27.

(27) Konto-Ghiorghi Y, Mairey E, Mallet A, Dumenil G, Caliot E, Trieu-Cuot P, et al. Dual role for pilus in adherence to epithelial cells and biofilm formation in Streptococcus agalactiae. PLoS Pathog 2009 May;5(5):e1000422.

(28) Sharma P, Lata H, Arya DK, Kashyap AK, Kumar H, Dua M, et al. Role of pilus proteins in adherence and invasion of Streptococcus agalactiae to the lung and cervical epithelial cells. J Biol Chem 2013 Feb 8;288(6):4023-4034.

(29) Spellerberg B, Rozdzinski E, Martin S, Weber-Heynemann J, Schnitzler N, Lutticken R, et al. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of Streptococcus agalactiae to human laminin. Infect Immun 1999 Feb;67(2):871-878.

(30) Schubert A, Zakikhany K, Pietrocola G, Meinke A, Speziale P, Eikmanns BJ, et al. The fibrinogen receptor FbsA promotes adherence of Streptococcus agalactiae to human epithelial cells. Infect Immun 2004 Nov;72(11):6197-6205.

(31) Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 2000 Oct;96(4):498-503.

(32) Brown CK, Gu ZY, Matsuka YV, Purushothaman SS, Winter LA, Cleary PP, et al. Structure of the streptococcal cell wall C5a peptidase. Proc Natl Acad Sci U S A 2005 Dec 20;102(51):18391-18396.

(33) Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK. Binding and invasion of HeLa and MRC-5 cells by Streptococcus agalactiae. Microbiology 2002 Dec;148(Pt 12):3921-3931.

(34) Baron MJ, Filman DJ, Prophete GA, Hogle JM, Madoff LC. Identification of a glycosaminoglycan binding region of the alpha C protein that mediates entry of group B Streptococci into host cells. J Biol Chem 2007 Apr 6;282(14):10526-10536.

(35) Bolduc GR, Madoff LC. The group B streptococcal alpha C protein binds alpha1beta1-integrin through a novel KTD motif that promotes internalization of GBS within human epithelial cells. Microbiology 2007 Dec;153(Pt 12):4039-4049.

(36) Seifert KN, McArthur WP, Bleiweis AS, Brady LJ. Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein-protein interactions. Can J Microbiol 2003 May;49(5):350-356.

(37) Pritchard DG, Lin B, Willingham TR, Baker JR. Characterization of the group B streptococcal hyaluronate lyase. Arch Biochem Biophys 1994 Dec;315(2):431-437.

(38) Milligan TW, Baker CJ, Straus DC, Mattingly SJ. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B streptococci. Infect Immun 1978 Sep;21(3):738-746.

(39) Sa de Sao Jose A, Miyazaki NH, Hirata R,Jr, Mattos-Guaraldi AL, Nagao PE. Intracellular viability in human non-polarized respiratory epithelial 16 HBE 14o- cells by group B Streptococcus serotype III clinical isolates presenting 162-kb and 183-kb virulence markers. Int J Mol Med 2006 Mar;17(3):533-538.

(40) Wang B, Yurecko RS, Dedhar S, Cleary PP. Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. Cell Microbiol 2006 Feb;8(2):257-266.

(41) Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science 2001 Nov 23;294(5547):1708-1712.

(42) Hao H, Naomoto Y, Bao X, Watanabe N, Sakurama K, Noma K, et al. Focal adhesion kinase as potential target for cancer therapy (Review). Oncol Rep 2009 Nov;22(5):973-979.

(43) Hehlgans S, Haase M, Cordes N. Signalling via integrins: implications for cell survival and anticancer strategies. Biochim Biophys Acta 2007 Jan;1775(1):163-180.

(44) Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci U S A 1992 Jun 1;89(11):5192-5196.

(45) Schaller MD. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 2001 Jul 25;1540(1):1-21.

(46) Dunty JM, Gabarra-Niecko V, King ML, Ceccarelli DF, Eck MJ, Schaller MD. FERM domain interaction promotes FAK signaling. Mol Cell Biol 2004 Jun;24(12):5353-5368.

(47) Hamada K, Shimizu T, Matsui T, Tsukita S, Hakoshima T. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J 2000 Sep 1;19(17):4449-4462.

(48) Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2000 May;2(5):249-256.

(49) Schaller MD, Otey CA, Hildebrand JD, Parsons JT. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J Cell Biol 1995 Sep;130(5):1181-1187.

(50) Reiske HR, Kao SC, Cary LA, Guan JL, Lai JF, Chen HC. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. J Biol Chem 1999 Apr 30;274(18):12361-12366.

(51) Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, et al. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature 1996 Jan 4;379(6560):91-96.

(52) Burnham CA, Shokoples SE, Tyrrell GJ. Invasion of HeLa cells by group B streptococcus requires the phosphoinositide-3-kinase signalling pathway and modulates phosphorylation of host-cell Akt and glycogen synthase kinase-3. Microbiology 2007 Dec;153(Pt 12):4240-4252.

(53) Burnham CA, Shokoples SE, Tyrrell GJ. Invasion of HeLa cells by group B streptococcus requires the phosphoinositide-3-kinase signalling pathway and modulates phosphorylation of host-cell Akt and glycogen synthase kinase-3. Microbiology 2007 Dec;153(Pt 12):4240-4252.

(54) Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005 Dec;4(12):988-1004.

(55) Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 2009 Aug;8(8):627-644.

(56) Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, et al. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. J Infect Dis 2009 Jan 1;199(1):108-115.

(57) Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem 1980 Jun;107(2):519-527.

(58) Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995 Dec 21-28;378(6559):785-789.

(59) Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002 Dec 12;420(6916):629-635.

(60) Lee SH, Dominguez R. Regulation of actin cytoskeleton dynamics in cells. Mol Cells 2010 Apr;29(4):311-325.

(61) Burnham CA, Shokoples SE, Tyrrell GJ. Rac1, RhoA, and Cdc42 participate in HeLa cell invasion by group B streptococcus. FEMS Microbiol Lett 2007 Jul;272(1):8-14.

(62) Shin S, Paul-Satyaseela M, Lee JS, Romer LH, Kim KS. Focal adhesion kinase is involved in type III group B streptococcal invasion of human brain microvascular endothelial cells. Microb Pathog 2006 Oct-Nov;41(4-5):168-173.

(63) Lemire P, Houde M, Lecours MP, Fittipaldi N, Segura M. Role of capsular polysaccharide in Group B Streptococccus interactions with dendritic cells. Microbes Infect 2012 Oct;14(12):1064-1076.

(64) Burnham CA. Interaction of the Group B Streptococci with Epithelial Cells: Host- Cell Signal Transduction Pathways in Group B Streptococcal Invasion and the Role of Surface Associated Group B Streptococcal Phosphoglycerate Kinase in Pathogenesis. 2007.

(65) Marques MB, Kasper DL, Pangburn MK, Wessels MR. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun 1992 Oct;60(10):3986-3993.

(66) Martin TR, Ruzinski JT, Rubens CE, Chi EY, Wilson CB. The effect of type-specific polysaccharide capsule on the clearance of group B streptococci from the lungs of infant and adult rats. J Infect Dis 1992 Feb;165(2):306-314.

(67) Rajagopal L. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiol 2009 Mar;4(2):201-221.

(68) Wessels MR, Rubens CE, Benedi VJ, Kasper DL. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc Natl Acad Sci U S A 1989 Nov;86(22):8983-8987.

(69) Bolduc GR, Baron MJ, Gravekamp C, Lachenauer CS, Madoff LC. The alpha C protein mediates internalization of group B Streptococcus within human cervical epithelial cells. Cell Microbiol 2002 Nov;4(11):751-758.

(70) Vallejo JG, Baker CJ, Edwards MS. Interleukin-6 production by human neonatal monocytes stimulated by type III group B streptococci. J Infect Dis 1996 Aug;174(2):332-337.

(71) Money DM, Dobson S, Canadian Paediatric Society, Infectious Diseases Commitee. The prevention of early-onset neonatal group B streptococcal disease. J Obstet Gynaecol Can 2004 Sep;26(9):826-840.

(72) Jordan HT, Farley MM, Craig A, Mohle-Boetani J, Harrison LH, Petit S, et al. Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. Pediatr Infect Dis J 2008 Dec;27(12):1057-1064.

(73) Shore EM, Yudin MH. Choice of antibiotic for group B streptococcus in women in labour based on antibiotic sensitivity testing. J Obstet Gynaecol Can 2012 Mar;34(3):230-235.

(74) 186-Women Receiving Group B*Streptococcus*Serotype III Tetanus Toxoid (GBS III-TT) Vaccine Have Reduced Vaginal and Rectal Acquisition of GBS Type III; October 30, 2009; Infectious Diseases Society of America (IDSA) 47th Annual Meeting.; 2009.

(75) Baker CJ, Paoletti LC, Wessels MR, Guttormsen HK, Rench MA, Hickman ME, et al. Safety and immunogenicity of capsular polysaccharide-tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. J Infect Dis 1999 Jan;179(1):142-150.

(76) Baker CJ, Paoletti LC, Rench MA, Guttormsen HK, Edwards MS, Kasper DL. Immune response of healthy women to 2 different group B streptococcal type V capsular polysaccharide-protein conjugate vaccines. J Infect Dis 2004 Mar 15;189(6):1103-1112.

(77) Edwards MS, Lane HJ, Hillier SL, Rench MA, Baker CJ. Persistence of functional antibodies to group B streptococcal capsular polysaccharides following

immunization with glycoconjugate vaccines. Vaccine 2012 Jun 13;30(28):4123-4126.

(78) Cheng Q, Debol S, Lam H, Eby R, Edwards L, Matsuka Y, et al. Immunization with C5a peptidase or peptidase-type III polysaccharide conjugate vaccines enhances clearance of group B Streptococci from lungs of infected mice. Infect Immun 2002 Nov;70(11):6409-6415.

(79) Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, et al. Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science 2005 Jul 1;309(5731):148-150.

(80) Henderson B, Martin A. Bacterial Moonlighting Proteins and Bacterial Virulence. Curr Top Microbiol Immunol 2011 Dec 6.

(81) Pancholi V, Fischetti VA. Alpha-Enolase, a Novel Strong Plasmin(ogen) Binding Protein on the Surface of Pathogenic Streptococci. J Biol Chem 1998 Jun 5;273(23):14503-14515.

(82) Pancholi V, Fischetti VA. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J Exp Med 1992 Aug 1;176(2):415-426.

(83) Winram SB, Lottenberg R. The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehyde-3-phosphate dehydrogenase. Microbiology 1996 Aug;142 (Pt 8)(Pt 8):2311-2320.

(84) Boel G, Jin H, Pancholi V. Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. Infect Immun 2005 Oct;73(10):6237-6248.

(85) Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, et al. Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. PLoS One 2012;7(1):e29963.

(86) Madureira P, Baptista M, Vieira M, Magalhaes V, Camelo A, Oliveira L, et al. Streptococcus agalactiae GAPDH is a virulence-associated immunomodulatory protein. J Immunol 2007 Feb 1;178(3):1379-1387.

(87) Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, Blom AM. Enolase of Streptococcus pneumoniae binds human complement inhibitor C4b-binding protein and contributes to complement evasion. J Immunol 2012 Oct 1;189(7):3575-3584.

(88) Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S. alpha-Enolase of Streptococcus pneumoniae induces formation of neutrophil extracellular traps. J Biol Chem 2012 Mar 23;287(13):10472-10481.

(89) Encheva V, Gharbia SE, Wait R, Begum S, Shah HN. Comparison of extraction procedures for proteome analysis of Streptococcus pneumoniae and a basic reference map. Proteomics 2006 Jun;6(11):3306-3317.

(90) Burnham CA, Shokoples SE, Tyrrell GJ. Phosphoglycerate kinase inhibits epithelial cell invasion by group B streptococci. Microb Pathog 2005 May-Jun;38(5-6):189-200.

(91) Boone TJ, Tyrrell GJ. Identification of genes affecting expression of phosphoglycerate kinase on the surface of group B streptococcus. Can J Microbiol 2012 Apr;58(4):433-441.

(92) Antikainen J, Kuparinen V, Lahteenmaki K, Korhonen TK. pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of Lactobacillus crispatus with the cell wall and lipoteichoic acids. J Bacteriol 2007 Jun;189(12):4539-4543.

(93) Feltcher ME, Braunstein M. Emerging themes in SecA2-mediated protein export. Nat Rev Microbiol 2012 Nov;10(11):779-789.

(94) Mistou MY, Dramsi S, Brega S, Poyart C, Trieu-Cuot P. Molecular dissection of the secA2 locus of group B Streptococcus reveals that glycosylation of the Srr1 LPXTG protein is required for full virulence. J Bacteriol 2009 Jul;191(13):4195-4206.

(95) Sheen TR, Jimenez A, Wang NY, Banerjee A, van Sorge NM, Doran KS. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. J Bacteriol 2011 Dec;193(24):6834-6842.

(96) Samen U, Eikmanns BJ, Reinscheid DJ, Borges F. The surface protein Srr-1 of Streptococcus agalactiae binds human keratin 4 and promotes adherence to epithelial HEp-2 cells. Infect Immun 2007 Nov;75(11):5405-5414.

(97) Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, Msadek T, et al. Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease. Mol Microbiol 2002 Sep;45(6):1499-1513.

(98) Lenz LL, Mohammadi S, Geissler A, Portnoy DA. SecA2-dependent secretion of autolytic enzymes promotes Listeria monocytogenes pathogenesis. Proc Natl Acad Sci U S A 2003 Oct 14;100(21):12432-12437.

(99) Bergmann S, Hammerschmidt S. Fibrinolysis and host response in bacterial infections. Thromb Haemost 2007 Sep;98(3):512-520.

(100) Wiles KG, Panizzi P, Kroh HK, Bock PE. Skizzle is a novel plasminogen- and plasmin-binding protein from Streptococcus agalactiae that targets proteins of human fibrinolysis to promote plasmin generation. J Biol Chem 2010 Jul 2;285(27):21153-21164.

(101) Magalhaes V, Veiga-Malta I, Almeida MR, Baptista M, Ribeiro A, Trieu-Cuot P, et al. Interaction with human plasminogen system turns on proteolytic activity in Streptococcus agalactiae and enhances its virulence in a mouse model. Microbes Infect 2007 Sep;9(11):1276-1284.

(102) Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, et al. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. Proc Natl Acad Sci U S A 2002 Sep 17;99(19):12391-12396.

(103) Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, Chhatwal GS, et al. Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of Streptococcus pneumoniae. Mol Microbiol 2003 Jul;49(2):411-423.

(104) Oswald N. 2007; Available at: http://bitesizebio.com/articles/pin-pointing-dna-ligation-problems/. Accessed March/12, 2013.

(105) Gel Electrophoresis of Proteins
A Practical Approach . Second ed. New York: Oxford University Press; 1990.

(106) Bucher T. Phosphoglycerate kinase from brewer's yeast : d-l,3-diphosphoglycerate + ADP d-3-phosphoglycerate + ATP. Methods in Enzymology 1955;1:415.

(107) Jin H, Song YP, Boel G, Kochar J, Pancholi V. Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. J Mol Biol 2005 Jul 1;350(1):27-41.